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Structure/function Studies Of Trypanosome Variant Surface Glycoproteins

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STRUCTURE/FUNCTION STUDIES OF TRYPANOSOME VARIANT SURFACE GLYCOPROTEINS

by

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ABSTRACT

Antigenic variation is manifested by many prominent infectious organisms in adaptive response to host immunity. However, polymorphism in antigenic structure is often limited by the necessity to maintain functionality of the antigen. It follows that it may be possible to design molecularly defined vaccines capable of accounting for variable antigenic phenotype. For this reason, the focus of the present thesis was to develop a model system using trypanosome variant surface glycoprotein (VSG) for examining the effects of amino acid sequence variation on the specificity of antigenic determinants.

In order to assess the suitability for a model system of four VSGs (isoVAT VSGs) known to share common surface-exposed epitopes, a variety of tests were utilized to elucidate structural and immunochemical properties. isoVAT VSGs were subjected to structural analysis using SDS PAGE, N-glycanase digestion, peptide mapping and amino acid analysis. Results disclosed structural similarities but also distinctions. Furthermore, immunochemical characteristics examined with polyclonal and monoclonal antibodies suggest significant antigenic homology but non-identity. Two novel strategies were developed to map antigenic determinants recognized by monoclonal antibodies. Relative distribution of antigenic sites was determined by probing VSG peptides resolved on western blots and precise locations (ie. to as little as six residues) of epitopes were elucidated using deletion analysis of expressed recombinant VSG.

Comparison of the aligned isoVAT VSG sequences revealed the presence of amino acid substitutions occurring in antigenic regions. Among the most radical replacements having no effect on antigenic specificity were those involving proline and others capable of creating significant charge differences. The only site for which specificity was affected by replacements displayed significant charge effect differences at two substituted positions.

Antigenic sites revealed in this study were positioned on a model VSG structure (Freymann *et al.*, 1990). Results provide the first evidence for positions of regions not exposed on the surface of the living trypanosome. Furthermore, findings suggest structural differences may exist between isolated VSG and VSG organized in the trypanosome coat.

Results of this thesis contribute to our understanding of how antigenic polymorphism can obstruct immune recognition and how the African trypanosome survives in its mammalian host.

DEDICATION

This PhD thesis is dedicated to my Grandmother,

Mrs Jean Carruthers, for showing me that an inquisitive

mind is sustenance for vivacity.

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I would like to sincerely thank my supervisor, Dr Michael W. Clarke for his endless patience and careful guidance. Although an active mentor in the lab, his passive teachings about practically all aspects of life will long be with me. Other members of my advisory committee, Dr Wayne Flintoff and Dr Stan Dunn, also deserve acknowledgement for offering many excellent suggestions and providing different perspectives on my project. Many thanks also to the rest of the "Clarkonians" (Terry, Teresa, Gino, and Ken) for being so tolerant and for not calling the authorities on countless occasions.

Most of all I must express my deepest love and appreciation for my wife, Cindy, for being so remarkably understanding under the most trying circumstances.

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LIST OF ABBREVIATIONS[1]

Abbreviation	Description
VSG	Variant Surface Glycoprotein
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
isoVAT	Iso Variant Antigenic Type
DNA	Deoxyribonucleic Acid
CDR	Complementary Determining Regions
VAT	Variant Antigenic Type
WaTat	Washington Trypanosome Antigenic Type
T.brucei	*Trypanosoma brucei*
PI PLC	Phosphatidyl Inositol Specific Phospholipase C
HIV	Human Immunodeficiency Virus
FMDV	Foot and Mouth Disease Virus
SV8	Staphylococcal V8 Protease
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetate
FBS	Fetal Bovine Serum
2-ME	2-Mercaptoethanol
DEAE	Diethylaminoethyl
PBS	Phosphate Buffered Saline
PSG	Phosphate Buffered Saiine + Glucose
mfVSG	Membrane Form Variant Surface Glycoprotein
sVSG	Soluble Form Variant Surface Glycoprotein
cmVSG	Carboxymethylated Variant Surface Glycoprotein
MAb	Monoclonal Antibody
GPI	Glycosyl Phosphatidyl Inositol
HPLC	High Performance Liquid Chromatography
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
Da	Daltons
KDa	Kilodaltons
M_r	Relative Molecular Weight
STD	Standard
ABTS	2,2'-azino-bis (3-ethylbenz-thiazoline sulfonic acid)
DAB	Diaminobenzidine
SA HRP	Streptavidin Horse Radish Peroxidase
NRS	Normal Rabbit Serum
NMS	Normal Mouse Serum
ND	Not Determined
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant

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[1]listed in approximate order of appearance

list of abbreviations cont'd

PBS-T	Phosphate Buffered Saline + Tween
ELISA	Enzyme Linked Immunosorbant Assay
RNAse	Ribonuclease
dNTP	Deoxynucleotide Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
DNAse	Deoxyribonuclease
DMSO	Dimethyl sulfoxide
Amp	Ampicillin
TBE	Tris Borate EDTA Buffer
IPTG	isopropyl-Beta-D-thiogalactopyranoside
LB	Luria Broth
TE	Tris EDTA Buffer
dGTP	Deoxy Guanosine Triphosphate
dATP	Deoxy Adenosine Triphosphate
dTTP	Deoxy Thymosine Triphosphate
dCTP	Deoxy Cytosine Triphosphate
ddGTP	Dideoxy Guanosine Triphosphate
ddATP	Dideoxy Adenosine Triphosphate
ddTTP	Dideoxy Thymosine Triphosphate
ddCTP	Dideoxy Cytosine Triphosphate
TEMED	N,N,N',N',-tetramethylethylene diamine
RIA	Radioimmunoassay
E.coli	*Escherichia coli*
cDNA	Complementary Deoxyribonucleic Acid
rVSG	Recombinant Variant Surface Glycoprotein
Exo III	Exonuclease III
bp	Base Pairs
AI	Antigenic Index
$O.D._{MAX}$	Maximum Optical Density
aa	Amino Acid
3D	Three Dimensional

Chapter 1

1.1 General Introduction

Technological advances in the past fifteen years have rapidly elevated our level of understanding of the molecular basis of immunity to the pathogens that plague our existence and the livelihood of our co-inhabitants. Among the most powerful were the development of monoclonal antibodies and recombinant DNA techniques, including DNA sequencing. These advances, above all others, have been responsible for the remarkable progress being made in the development of high tech vaccines against infectious organisms for which traditional vaccine strategies have failed. However, recent accomplishments only hold marvel for us until we are reminded of the challenging tasks we still face. Paramount among the mandates, is the development of effective immunoprophylactics against malaria and AIDS. Likely not by coincidence, these eminent pathogens show common ability to exhibit antigenic variation. This will undoubtedly complicate the production of respective immunoprophylactic agents.

Suitable models are required for examining the practical limits of antigenic variation as an immune evasion strategy. Variant surface glycoprotein, the subject of this thesis, may indeed be the most felicitous model available since it itself is the mediator of antigenic variation for the master of immune evasion - the African trypanosome. Results presented in this study using the VSG model, contribute to an experimental basis upon which future vaccines can be designed to account for the exquisite adaptability of the most devastating pathogens.

1.2 Antigen/Antibody Interactions

Of obvious importance to the design of effective vaccines is a complete understanding of the effector recognition mechanisms of the immune system. Particularly for extracellular pathogens, more amenable to clearing by humoral responses, this involves delineating the nature of the antibody/antigen interaction. The following is a brief synopsis of present knowledge in this regard.

Binding sites or paratopes of antibodies are composed of six hypervariable loops or complementarity determining regions (CDRs), which directly interact with antigenic determinants through Van der Waals bonds, hydrogen bonds, Coulombic bonds, hydrophobic interactions and Ca^{2+} bridges. Also, proper fit between the paratope and its complementary antigenic determinant is of extreme importance for maximum affinity. Although paratopes were initially believed to take the shape of a pocket, crystallographic studies on antigen/antibody complexes have shown that paratopes can have relatively flat binding surfaces with extensive interdigitation occurring between CDRs and the antigenic determinant (Amit *et al.*, 1986).

Protein antigenic determinants (for review see van Regenmortel, 1988) are of two types: linear (a.k.a. segmental), which are composed of contiguous residues in one stretch of the polypeptide and non-linear (a.k.a. non-segmental or topographically assembled), which can involve residues distally separated in the sequence but brought together in the mature folded protein. Non-linear epitopes are more difficult to characterize with respect to the identification of residues

comprising the site, since any manipulations which affect the structural integrity of the antigen often results in destruction of the determinant. In contrast, linear epitopes are more amenable to detailed analysis since their integrity is maintained during manipulations required for localization. Because of their linear nature, these antigenic determinants can be artificially reproduced in the form of a synthetic peptide. In fact significant optimism regarding the use of synthetic peptides as vaccines has been generated in recent years (reviewed in Riveau and Audibert, 1990). Fuelling the optimism was the discovery that antibodies prepared against a peptide representing a linear epitope could bind to the corresponding determinant on tne native antigen and vice versa (Atassi, 1984; Wilson *et al.*, 1984; Geysen *et al.*, 1985). However, it was not clear how antibodies prepared against the whole antigen could bind to a synthetic epitope, which, in solution, probably did not stably adopt the same conformation it assumes in the native protein. A theory proposed by Geysen and his co-workers (Getzoff *et al.*, 1987), although originally formulated for whole antigens, perhaps offers an explanation for the synthetic peptide enigma. Their theory of induced fit postulates that antibody binding occurs in a two step process. First, low affinity interaction initially brings the antigenic determinant and the antibody paratope into close proximity, then the determinant undergoes minor conformational changes allowing tight fit with the antibody paratope. In this manner, maximum bonding occurs between residues in the antigenic determinant and the CDRs of the antibody.

Additionally, synthetic peptides have been useful for characterizing the sub-

structure of linear epitopes by substitution analyses. Geyson and his colleagues (Getzoff *et al.*, 1987) also discovered that some residues in a linear epitope could be replaced with all other nineteen amino acids yet residues at other positions were completely non-replaceable. This lead to the postulation that not all residues in a linear determinant actually made contact with residues in the antibody CDRs. This suggested that non-contact residues may simply act as a chassis or framework for contact residues. Furthermore, the results of replaceability analyses emphasised the prospect that only a few key residues may be responsible for binding specificity and affinity. Revealing some insight into the molecular basis of serotypic conversion resulting from antigenic drift and antigenic shift, Houghten and his associates (Houghten *et al.*, 1986; Rowlands *et al.*, 1983) further demonstrated the importance of one or a few key residues in determining the antigenic specificity of linear determinants. What remains unclear, however, is whether experimental results generated in an extremely artificial system, such as using synthetic peptides, can be applied to practical situations. Additionally, only a small number of antigenic sites have been subjected to detailed substitutional analysis, therefore, our understanding regarding replaceability of residues within antigenic sites remains incomplete.

1.3 Design of Molecularly Defined Vaccines

Although our level of technological sophistication has advanced tremendously since the first vaccine was developed by Edward Jenner in 1795, it has been somewhat difficult to match the remarkable success of the most famous

prototypical vaccines such as Jenner's smallpox vaccine and Sabin's polio vaccine. Most would agree, however, that we presently face much more challenging problems with respect to vaccine development than those encountered by the pioneering vaccine engineers.

Both the Jenner and Sabin vaccines are examples of live attenuated vaccines, however, successes have been achieved with inactivated pathogens (Salk-polio, Rabies) as well. For these first generation vaccines, attenuation and inactivation were the only strategies employed for eliminating or at least reducing the pathogenicity and virulence of the infectious organism. Although highly successful in many cases, these strategies are not without problems. For example, attenuated vaccines carry the possibility of spreading virulent wild type organisms generated in the immunized individual and in most cases, guaranteeing complete inactivation is difficult or impossible for inactivated vaccines. In addition, these vaccine production strategies cannot be applied to many pathogens. Reasons include (1) inability to grow sufficient amounts of the pathogen; (2) inability to isolate avirulent strains; (3) poor protection against some inactivated pathogens due to modification (denaturation etc.) of critical antigens; (4) immunization with whole organisms can result in ineffective or poor immune responses due to (a) the presence of antigens capable of suppressing immune responses and (b) misdirection of the response ie. towards non-protective antigens rather than protective ones.

Problems associated with first generation vaccines provided the impetus for

developing vaccines that may circumvent some of the pitfalls of their predecessors (reviewed in Brown, 1990). Unlike prototypic vaccines, these second generation vaccines are subunit vaccines, that is, they are usually composed of only one antigen or a portion thereof, not the entire organism. Furthermore many are molecularly defined, in the sense that the principle protective antigens used have been characterized in some detail. Circumventing problems (1) and (2), advances in foreign gene expression in prokaryotic and eukaryotic systems has allowed isolation of sufficient quantities of material from non-cultivable pathogens. Furthermore problems (3) and (4) are eliminated due to the subunit nature of these high tech vaccines. One significant problem that has plagued subunit vaccines is poor immunogenicity. That is, they often evoke responses insufficient for protective immunity. For this reason, a considerable amount of research has been directed towards finding means of enhancing immunogenicity of potentially protective antigens. To this end, a number of innovative vaccine delivery strategies have been developed. These include the exploitation of whole organisms such as viruses: vaccinia and polio; and bacteria: mycobacteria (Mackett *et al.*, 1985; Jacobs *et al.*, 1987; and Hogle, 1988 respectively). In addition, incorporation of protective antigens into molecular delivery vehicles such as liposomes (Derksen and Scherphof, 1985), proteosomes (Lowell *et al.*, 1988) and iscoms (Morein *et al.*, 1990) has been reported. Also documented (Barber and Carayanniotis, 1990), is the use of monoclonal antibodies coupled with antigen for specific targeting and delivery to immune recognition cells. Additionally, particularly for synthetic peptide

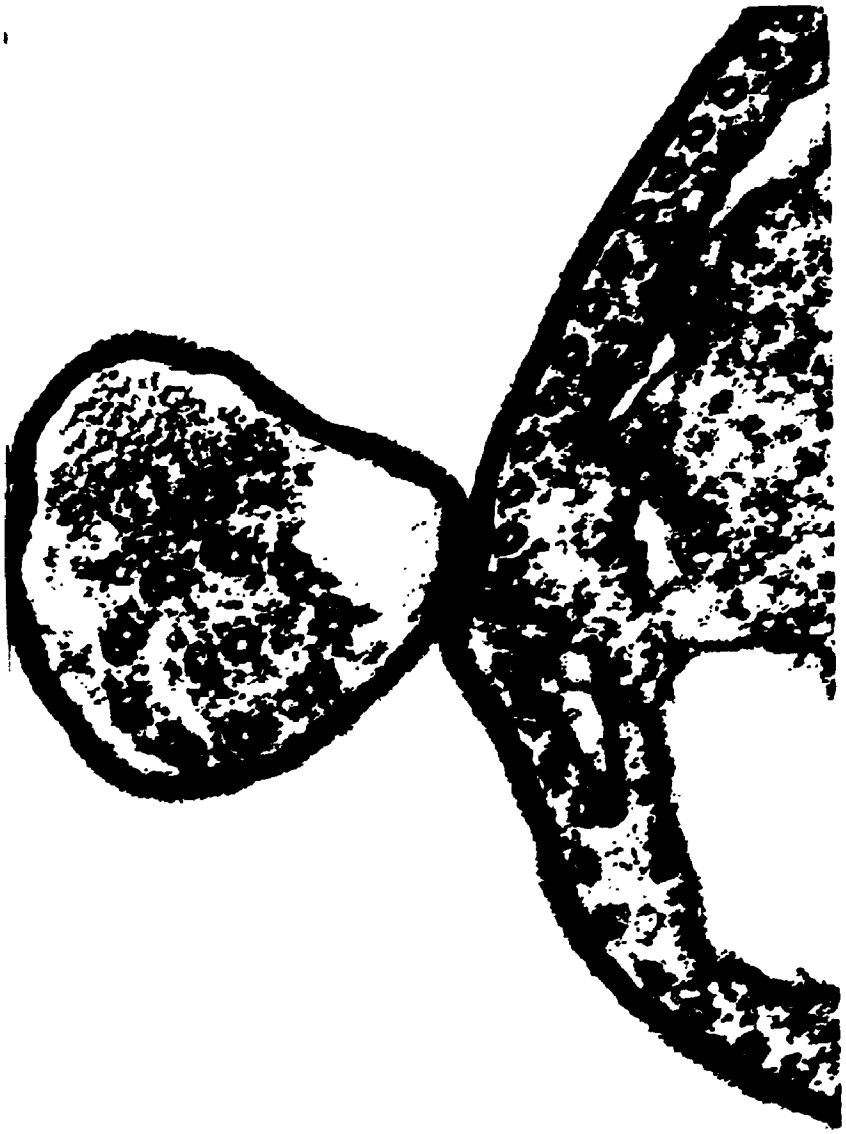
vaccines, co-presentation of T-helper epitopes with neutralizing B-cell epitopes, has been shown to increase immunogenicity significantly (eg. Hart *et al.*, 1990).

Although considerable progress has been made toward solving the problem of poor immunogenicity of subunit vaccines, we still face the problem of how to design molecularly defined vaccines capable of accounting for antigenic variation. Since subunit vaccines are composed of one or a few epitopes, they are presumably more susceptible to becoming ineffectual due to the consequences of mutation than traditional vaccines. For this reason a better understanding of the effects of amino acid substitutions on the specificity of antigenic determinants is required.

1.4 An Overview of Trypanosome Antigenic Variation and VSG Structure/Function

Trypanosomes survive in their mammalian host using the immune evasion strategy of antigenic variation. This is accomplished by periodically changing serotype or variant antigenic type (VAT) by antigenic switching. The structure responsible for antigenic variation was first identified as an electron dense layer (surface coat), completely covering the plasma membrane of animal infective form (bloodstream form) trypanosomes (Vickerman, 1969; Vickerman and Luckins, 1969)(Figure 1.1). As shown in classic experiments by Cross (Cross, 1975) the surface coat is apparently composed of a single antigen, the variant surface glycoprotein (VSG). VSG is entirely responsible for serotype specificity and it is the only membrane antigen recognized by the host immune response during a natural infection (Cross, 1975). To this day the only known function of the molecule is to

Figure 1.1: Transmission electron micrograph of a thin section preparation of bloodstream trypanosomes. Illustrated is the electron-dense surface coat that completely covers the cell body (shown to the right) and the flagellum (left). Magnification X 132,500. Modified from Shapiro and Pearson (1986).



act as a "dummy" antigen, fielding the host immune response until a switch occurs, thus allowing survival of the parasite (Vickerman, 1989).

Although very little is currently known about regulation of VSG gene expression, mechanisms of chromosomal rearrangements which form the genetic basis of antigenic variation have been characterized to some degree. The most common mechanism involves a gene conversion event during which the "basic copy" of a VSG gene, most often located in a chromosome internal site, is duplicated and transposed into a telomeric expression site, generating an "expression linked copy" (ELC)(Pays *et al.*, 1983). The transposed copy enters the expression site by homologous recombination with the ELC of a previously expressed VSG occupying the site. Although the recombinational event usually involves homologous regions which exist both upstream and downstream of the coding region, recombinations can occur within the VSG open reading frame provided some homology exists between the resident gene and the incoming gene. Such partial gene conversions result in the formation of hybrid VSG genes which are thought to be of significance for the evolution of antigenic variation and the generation of novel VSG repertoires.

Initial immunochemical studies (Pearson *et al.*, 1980) revealed the presence of two types of antigenic determinants on the VSG that differ in accessibility when the antigen is present in the surface coat. Monoclonal antibodies were used to determine that some epitopes are accessible on the trypanosome surface (as indicated by the results of immunofluorescence assays using living trypanosomes),

while others are inaccessible or cryptic when the VSG is assembled into surface coat. However, cryptic epitopes can be exposed if trypanosomes are fixed prior to immunofluorescence. Except among closely related VSGs (eg. WaTat VSGs, see below), no antigenic cross-reactivity of protein epitopes has been documented. Although this is not surprising for surface exposed epitopes on VSGs, it remains uncertain as to why cryptic antigenic determinants are not conserved to any degree.

Owing to its ease of purification in large quantities, the VSG has been examined in some detail with regards to structure, since it was first discovered in 1975. VSGs range in apparent molecular weight from 52 to 69 kDa and correspondingly vary in length from 450 to 500 amino acids for the mature polypeptide (Johnson and Cross, 1977). In the order of a dozen VSGs have been fully sequenced to date, in addition to a number of partial sequences available. Multiple sequence alignment of several VSG sequences demonstrated the presence of considerable homology near the mature C-terminus (Rice-Ficht *et al.*, 1981) and less obvious, yet still significant, homology in the N-terminal 30 amino acids (Olafson *et al.*, 1984). Overall, however, sequences show greatest divergence in the N-terminal two thirds of the polypeptide, hence this domain is often referred to as the hypervariable domain. Amongst this extensive sequence variation, however, lies a significant degree of conservation of cysteine residues both positionally and quantitatively (Turner, 1988). This presumably reflects an important role for this particular residue, perhaps in maintaining common tertiary structure

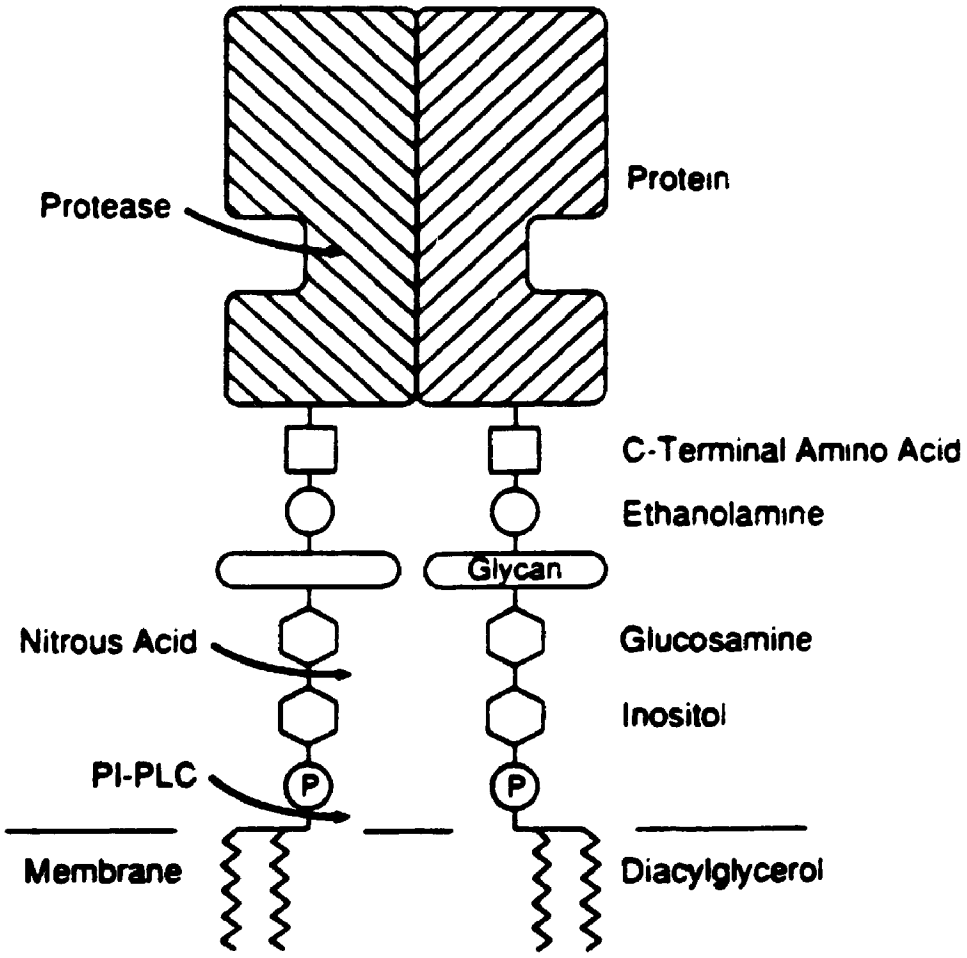
among VSGs.

All VSGs have at least one N-linked oligosaccharide which appears to be of the high mannose variety (Savage *et al.*, 1984) and is not immunogenic, probably by virtue of its structural similarity to carbohydrates of other eukaryotes, including its host. Furthermore, oligosaccharides are not accessible on the trypanosome surface as demonstrated by lectin affinity studies (Johnson and Cross, 1977). VSGs are anchored into the cell membrane by a C-terminal glycosyl phosphatidyl inositol (GPI) glycolipid (for review see Cross, 1990)(Figure 1.2) which contains highly conserved sugar residues making it antigenically cross-reactive among VSGs of different VATs. For this reason it commonly referred to as the cross-reactive determinant or CRD. Unfortunately, the CRD is not exposed on the trypanosome surface and, therefore, it cannot be exploited for immunoprophylactic intervention.

When VSGs are mildly treated with a protease such as trypsin, two major products result, suggesting the existence of two structural domains: an N-terminal domain (2/3 to 3/4 of the sequence) and a C-terminal domain (1/3 to 1/4 of the polypeptide). In addition to evidence for conservation of domains, spectral analyses demonstrate the presence of common secondary structure characteristics (ca. 50% alpha helix, 10-20% beta sheet and 30-40% random coil or beta turn), at least for *T. brucei* VSGs (Turner, 1988; Clarke *et al.*, 1988). Additional evidence for the idea that VSGs may exhibit common three dimensional structure was presented by Metcalf and his colleagues (Freyman *et al.*, 1984) when remarkable

Figure 1.2: Schematic representation of VSG structure and attachment to the membrane. VSG is illustrated as a two domain, somewhat elongated structure associated as a dimer. Also indicated is the partial structure of the glycolipid anchor and cleavage points for various reagents used to examine the structure. Modified from Turner (1988).

STRUCTURE OF VSG OF T.BRUCEI



similarity was observe between 6 Angstrom electron density maps of two unrelated VSGs. Furthermore, a detailed analysis of the crystal structure of one VSG (Freymann *et al.*, 1990) has resulted in the disclosure of several conserved structural motifs present in most VSGs sequenced to date. These motifs include (1) heptad periodicity corresponding to the two core alpha helices (2) conserved hydrophobic residues at 4 positions surrounding Cys^{15} and (3) a well conserved 5 amino acid sequence Gly-Arg-Ile-Asp-Glu (GRIDE). These sequence motifs are likely to be at least partially responsible for maintaining common VSG tertiary structure.

Regarding quaternary structure, VSGs are known to exist as dimers in solution (Auffret and Turner, 1981; Rehaber *et al.*, 1990) and probably exist in the same form in the surface coat, although cross-linking experiments result in the generation of oligomers of nearly all sizes, presumably reflecting high packing density. Other than evidence for high density packing, very little is known about VSG association in the surface layer. Also remaining unclear, is what portions of the polypeptide are accessible on the surface of the trypanosome.

With respect to structure/function, the VSG should, but clearly doesn't, have a dilemma; how to be functionally effective as an antigenic polymorph yet maintain common tertiary structure, which is presumably compulsory for proper packing and arrangement in the coat. Recent results generated from X-ray diffraction analysis of a crystallized VSG (Freymann *et al.*, 1990) shed considerable light upon this issue, however, our understanding is far from complete. Therefore, completely

solving the apparent paradox will undoubtedly advance our understanding of the dependency of higher order structure on amino acid sequence in polypeptides.

1.5 The WaTat Serodeme

Trypanosome variants having the capacity to express the same VSG repertoire are referred to as belonging to the same serodeme. In other words, a serodeme can be described as a family of trypanosome variants which differ in antigenic phenotype but are, for all intents and purposes, genotypically identical. Although serodemes occur naturally, they can be examined under artificial conditions in the laboratory by first cloning a parental variant then isolating subsequent variants generated as a result of antigenic switching. In this manner, one can isolate hundreds of variants which collectively represent the VAT repertoire of the serodeme.

In the early 1980s, A.F. Barbet and his associates at Washington State University generated a serodeme from stabilate EATRO 110 which was originally isolated from a Ugandan warthog. The so called WaTat (Washington Trypanosome antigenic type) serodeme, was initiated by cloning the parent, designated WaTat 1.1, several times in immunocompromised rats (thus avoiding the possibility of antigenic switch), until it produced a 99% homogeneous infection with respect to VAT. The parent was then used to infect an immunocompetent rabbit which developed a typical infection, characterized by periodic parasitaemic waves, each corresponding to expression of a different VAT. Trypanosomes were isolated at weekly intervals and stabilates made after expansion of each isolate in rats. These

stabilates were then used to initiate infections of Deer mice and subsequent populations were derived from isolates taken again at weekly intervals. These isolates (hundreds in total) were examined for surface fluorescence after treatment with polyclonal antibodies prepared against the parent variant WaTat 1.1 using an indirect immunofluorescence assay (IFA) on living cells. The objective was to isolate daughter variants which shared surface exposed epitopes with the parent WaTat 1.1. Isolates, positive in the IFA, were cloned once then re-examined for surface fluorescence. Three variants, designated WaTats 1.12, 1.13 and 1.14, were selected in this manner, bringing the total number of cross-reactive variants to four. These variants are collectively referred to as the WaTat isoVAT.

Evidence proving that WaTat 1.1 and WaTat 1.12 VSGs are not identical was presented in the same article describing the generation of the serodeme (Barbet *et al.*, 1982). It was shown that these two VSGs differed to varying degrees in isoelectric point, tryptic maps and immunochemical identity examined by monoclonal antibodies. WaTats 1.13 and 1.14 were not included in this study because they had not yet been selected at the time.

1.6 Rationale and Thesis Objectives

Antigenic shift, drift and variation pose serious problems to vaccine development and design. All types of pathogens have been shown to exhibit some degree of antigenic variation. These include viruses: HIV-I, influenza virus, lentivirus, FMDV and polio virus; bacteria: Niesseria and Borrelia; and parasites: trypanosomes, plasmodia and giardia (Air *et al.*,1989; Montelaro *et al.*, 1989; Rowlands

et al., 1983; Hovi, 1989; Meyer, 1089; Barstad *et al.*, 1985; Pays, 1985; Newbold *et al.*, 1984; and Glenney, 1987 respectively). Development of vaccines against many of these pathogens may require some means of accounting for and circumventing antigenic polymorphism. This can only be achieved with a greater understanding of how amino acid sequence variation affects serological specificity. With our current level of understanding in this regard it is impossible to determine if vaccines can be engineered to protect against a variety of antigenic mutants. Clearly, in cases of extreme antigenic variation, such as the African trypanosome, taking into account all antigenic variants appears to be an impossible task. However, with pathogens that exhibit only limited variation, the question of whether it is possible to engineer a multivalent vaccine which can protect against all serotypically distinct variants remains open.

Results generated from model systems that examine the relationship between amino acid substitutions and antigenic specificity may be of extreme value. Information collected initially on these model systems may be subsequently applied to individual practical situations in order to resolve any differences that might exist between systems.

Although an impressive amount of information has been generated from comprehensive replacement analyses using synthetic peptides, this approach has received considerable criticism of its extreme artificiality (eg. Laver *et al.*, 1990). In addition, these studies suffer from a lack of quality control, considering the side reactions that commonly occur during peptide synthesis. The fact remains that the

only completely definitive way to characterize antigenic determinants is by co-crystallization of antigen/antibody complexes followed by X-ray diffraction analysis. Obviously, however, this is an exceedingly arduous process to extensively examine substitutional effects, therefore artificial systems are necessary to generate basic knowledge in this regard.

We have elected to use closely related isomers of trypanosome variant surface glycoproteins in a model system for examining the effects of limited sequence variation on serospecificity. This system is somewhat less artificial than the synthetic peptide approach and, therefore, information generated may be of more practical use. Additionally, the results of this study have interesting implications regarding VSG structure and molecular organization in the trypanosome surface coat, which in turn contributes to our understanding of the African trypanosome's remarkably successful survival strategies.

Summarized, the primary objectives of this thesis are as follows:

(1) to characterize the structural and immunochemical relationships among VSGs exhibiting common surface exposed epitopes

(2) to determine position and structure of antigenic determinants recognized by monoclonal antibodies and by comparing sequences within these sites, determine the effects of amino acid substitutions on antigenic specificity

(3) to examine VSG structure and arrangement in the trypanosome surface coat using monoclonal antibodies as reagents.

Chapter 2 - Structural Studies of WaTat isoVAT VSGs

2.1 Introduction

At the time research was started on this thesis, relatively little was known about the WaTat isoVAT VSGs and virtually all information available was regarding variants 1.1 and 1.12 (Barbet *et al.*, 1982). With respect to the limited physical structure data, WaTat 1.1 and WaTat 1.12 VSGs were known to differ considerably in isoelectric points and tryptic peptide maps. However, amino acid compositions of these variants (Olafson *et al.*, 1984) suggested the existence of significant primary structure homologies. Furthermore, N-terminal sequence analysis of all four variants demonstrated that, at least in the first 30 or so amino acids, the sequences were identical at most of the positions (ca. 75%) but point substitutions were found to exist throughout. Given this limited information, my initial mandate was to further investigate the structural relationship existing among these variants, particularly including WaTat 1.13 and WaTat 1.14 VSGs.

Among the questions still remaining at the time were: are the relative molecular weights of the isoVAT VSGs sufficiently similar to indicate relatedness, are oligosaaccharides conserved among the VSGs and what is the full extent of amino acid sequence homologies and do they extend beyond the N-terminal region? Furthermore, it was important to have some information regarding physical structure in order to make comparisons to the results generated from immuno-chemical studies being performed concurrently.

Of the variety of techniques available, peptide mapping was chosen to

further examine primary structure homologies since this technique is rapid and relatively easy to perform.

Although the strategy of peptide mapping is a reliable means of investigating the degree of amino acid sequence homology existing between two or more proteins, a few notes on the interpretation of results generated by this technique are warranted. Particularly, when comparing fragment patterns generated with highly specific cleavage reagents, one can only make valid conclusions regarding structural homologies at cleavage site amino acids. For example, if CNBr cleavage resulted in identical fragment patterns for two different proteins, then it can be concluded that the positions of methionine residues are conserved. However, it is often assumed that observable homologies with respect to cleavage site residues are indicative of additional homologies at other positions. In addition, one must be careful when interpreting peptide maps of glycoproteins because, without experimental evidence, conservation of oligosaccharides, both positionally and quantitatively, cannot be assumed.

Presented in this chapter are the results of indirect methods used to examine structural homologies among isoVAT VSGs. These results form the basis for eventual complete delineation of the structural features of these VSGs.

2.2 Materials and Methods

2.2.1 Chemicals and Biochemicals

All standard laboratory chemicals were purchased as reagent or analytical grade or better. Isopropanol, methanol, acetic acid, formic acid, chloroform, glucose and sodium chloride were obtained from BDH. Sigma provided the cyclophosphamide, diethylaminoethyl (DEAE) 52 cellulose, iodoacetamide and Triton X-100, while dithiothreitol (DTT), staphylococcus V8 (SV8) protease and ethylenediamine tetra acetate (EDTA) were purchased from Boehringer Mannheim. Fetal bovine serum (FBS), N-glycanase and heparin were supplied by Flow, Genzyme and Organon Teknika respectively and Baker supplied the sodium phosphate reagents. 2-mercaptoethanol (2-ME) and zinc chloride. Rats were obtained from Charles River and glycerol and ammonium hydroxide were purchased from Fisher Scientific. Also, coomassie brilliant blue R-250 and sodium dodecyl sulfate (SDS) were supplied by Serva.

2.2.2 Origin and Storage of T.brucei Variants

The *T. brucei* variants used in this study are all members of the WaTat (Washington trypanosome antigenic type) serodeme which was derived from variant EATRO 110 (East African Trypanosomiasis Research Organization, Tororo, Uganda). Construction of the WaTat serodeme has been described previously (Barbet *et al.*, 1982). Trypanosome stocks were stored at -196°C under liquid nitrogen as semi-purified cells in freezing medium (90% FBS, 10% glycerol). Trypanosomes were semi-purified by centrifuging infected rat blood at 1000xg in

an American Scientific Products Omnifuge RT with a C1726-20 rotor for 10 minutes at 4°C. The buffy coat region containing the trypanosomes was aspirated and the parasites were typically diluted to $1.0x10^7$ cells/ml in freezing medium. 1 ml aliquots were frozen slowly overnight in cryotubes (Nunc) using a Taylor-Wharton Handi-Freeze freezing tray.

2.2.3 Growth and Purification of Parasites

Trypanosomes were grown in male or female Sprague-Dawley rats immunosuppressed with cyclophosphamide in order to prevent elimination of the VAT by host immune response. Briefly, on day 1, rats were each injected with 2.0 ml of a 25 mg/ml solution of cyclophosphamide in phosphate buffered saline (PBS, 57 mM sodium phosphate dibasic, 3 mM sodium phosphate monobasic and 44 mM sodium chloride pH 8.0). 24 hrs later (day 2), $1.0x10^6$ trypanosomes diluted in PSG (PBS + 1% (w/v) glucose) were injected per rat. When parasitaemia approached or attained $1.0x10^9$/ml (usually on day 4 or 5), rats were anaesthetized in chloroform vapour and blood was collected from the thoracic cavity after lacerating the heart. As the blood was collected, heparin (50 units/ml in PSG) was added to a final concentration of 10 units/ml ie. 1 part heparin solution to 4 parts blood. Blood was centrifuged as described in section 2.2.2 and the buffy coat was diluted 1:1 with PBS. Parasites were then purified from contaminating blood cells using a DEAE 52 cellulose column (dimensions varied according to the size of the preparation) equilibrated in PSG pH 8.0.

2.2.4 VSG Purification

Purified preparations of both membrane form (mfVSG) and soluble form (sVSG) VSGs were required as immunogens for production of MAbs. After purification, VSG preparations were stored as small aliquots (50-200 μl at 1 mg/ml) at -20°C.

2.2.4.1 HPLC Purification of Membrane Form VSG

mfVSG was purified using a Beckman high performance liquid chromatography instrument (HPLC) according to the method described by Clarke *et al.* (1984). Fractions corresponding to peaks on chromatograms were collected, concentrated using a Savant Speed Vac concentrator, then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Fractions containing purified mfVSG were pooled and protein concentration was determined by the method of Lowry *et al.* (1951).

2.2.4.2 Osmotic Shock Purification of Soluble VSG

The method used to purify sVSG is described in detail by Cross (Cross, 1975). Briefly, purified trypanosomes were pelleted and osmotically shocked by resuspending them to 2.0×10^9 cells/ml in 0.3 mM zinc acetate at 0°C. The temperature was maintained at 0°C for 4 minutes followed by centrifugation at 3000xg for 5 minutes at 4°C. The cells were resuspended with the same volume of 10 mM phosphate buffer pH 8.0 containing 0.1 mM TLCK and incubated at 37°C for 5 minutes followed by centrifugation at 10,000xg for 15 minutes. The supernatant was applied to a DEAE 52 cellulose column equilibrated in 10 mM sodium phosphate buffer pH 8.0 and the flow through fraction collected. After

elution from the DEAE column a portion of the VSG fraction was analyzed by SDS PAGE. VSG purity was such that further enrichment by isoelectric focusing (Cross, 1975) was deemed unnecessary.

2.2.5 Electrophoresis of Proteins and Protein Fragments

VSG and fragments derived from VSG, were subjected to SDS poly-acrylamide gel electrophoresis (SDS PAGE) using a BioRad mini-protean II electrophoresis unit. Dimensions of the gels were 8.5x7.0 cm, length by width. Samples were prepared for SDS PAGE by either dissolving the dry protein sample directly in SDS PAGE sample buffer or by adding an equal volume of 2X sample buffer (ie. if the protein sample was already in solution). Samples were heated to 100°C for 5 minutes, then centrifuged at 16,000xg for 2-3 minutes prior to loading. Most gels were stained with coomassie brilliant blue R-250 (0.05% w/v in 25% v/v isopropanol, 10% v/v acetic acid, 65% distilled water) for 30 minutes to overnight. This staining solution was routinely filtered through Whatman 3mm paper prior to use. Coomassie stained gels were destained using a solution composed of 35% methanol (v/v) and 10% acetic acid (v/v) in distilled water until backgrounds were clear. Occasionally, when increased sensitivity was required, SDS PAGE gels were stained with silver according to Merril (1990).

2.2.5.1 SDS PAGE of Proteins

SDS PAGE protein gels were prepared and electrophoresed according to the gel apparatus manufacturers instructions (BioRad), which essentially follow the procedure developed by Laemmli (1970). For both the determination of relative

molecular weights of WaTat VSGs and N-glycanase experiments, gels contained 10% acrylamide in the separating gel and 4% acrylamide in the stacking gel. Gels were electrophoresed at 150 constant volts until the bromophenol blue dye front reached the bottom. BioRad low molecular weight standards (14-93 kilodalton range) were used as reference sized markers for relative molecular weight determinations.

2.2.5.2 SDS PAGE of Protein Fragments

12-17% linear gradient SDS PAGE gels (Fling and Gregerson, 1986) were used to resolve low molecular weight VSG fragments in peptide mapping experiments. The Fling SDS PAGE system (Fling and Gregerson, 1986) is identical to the Laemmli (1970) system except stacking gels and running buffer contain twice as much Tris. VSG fragments were electrophoresed under constant voltage at 100 volts until the dye front had run completely off the bottom of the gel. A mixture of BioRad low molecular weight standards and Sigma peptide standards (CNBr cleaved horse heart myoglobin) were used as size markers for peptide gels.

2.2.6 Deglycosylation Experiments

Removal of N-linked oligosaacharides from WaTat VSGs was accomplished with N-glycanase at 10 units/ml in 0.1M sodium phosphate buffer (pH 6.5) containing 10 mM EDTA, 0.1% Triton X-100, O.5% SDS and 0.1% 2-mercapto-ethanol at 37°C for 18 hours.

2.2.7 Generation of VSG Fragments

2.2.7.1 Fragmentation with Cyanogen Bromide

In preparation for cleavage with CNBr, VSGs were reduced with DTT then carboxymethylated with iodoacetamide (Crestfield *et al.*1963). Progress of the carboxymethylation reaction was monitored by 2D SDS PAGE gels. CNBr cleavages were carried out with a 400 molar excess of CNBr with respect to methionyl residues in 70% (v/v) formic acid in the dark at 20°C for 24 hours. Samples were then lyophilized to dryness, reconstituted in distilled water, then lyophilized again. Finally, terminal methionyl residues in the lactone form were converted to the peptide form by treatment with 0.1% (w/v) ammonium hydroxide for 15 minutes.

2.2.7.2 Cleavage with Staphylococcus V8 Protease

Enzymatic cleavage of VSG with SV8 protease for SDS PAGE was performed in SDS PAGE sample buffer at 20°C for 5 hours with a 1:20 enzyme to substrate ratio. Final protein concentration was 1.0 mg/ml.

2.3 Results

2.3.1 VSG Purification

Purified VSG was required for use in structural studies and immunizations of mice and rabbits. Originally, it was decided to use mfVSG for mouse immunizations and subsequent production of MAbs - the justification being the possibility that, in addition to the primary goal of producing anti-polypeptide MAbs, anti-CRD MAbs might be created. Anti-CRD MAbs, of which none presently exist, would be of significant use for studying glycophosphatidyl inositol (GPI) anchored proteins. While no anti-CRD antibodies were isolated in this study, one anti-mfVSG MAb was produced (dn13A1).

It was decided to switch to purification of sVSG because this form could be isolated on a much larger scale with greater yields. As a result, sVSG was used for all of the structural studies presented in this chapter and for the production of all polyclonal and monoclonal antibodies with the exception of dn13A1.

2.3.1.1 mfVSG Purification by HPLC

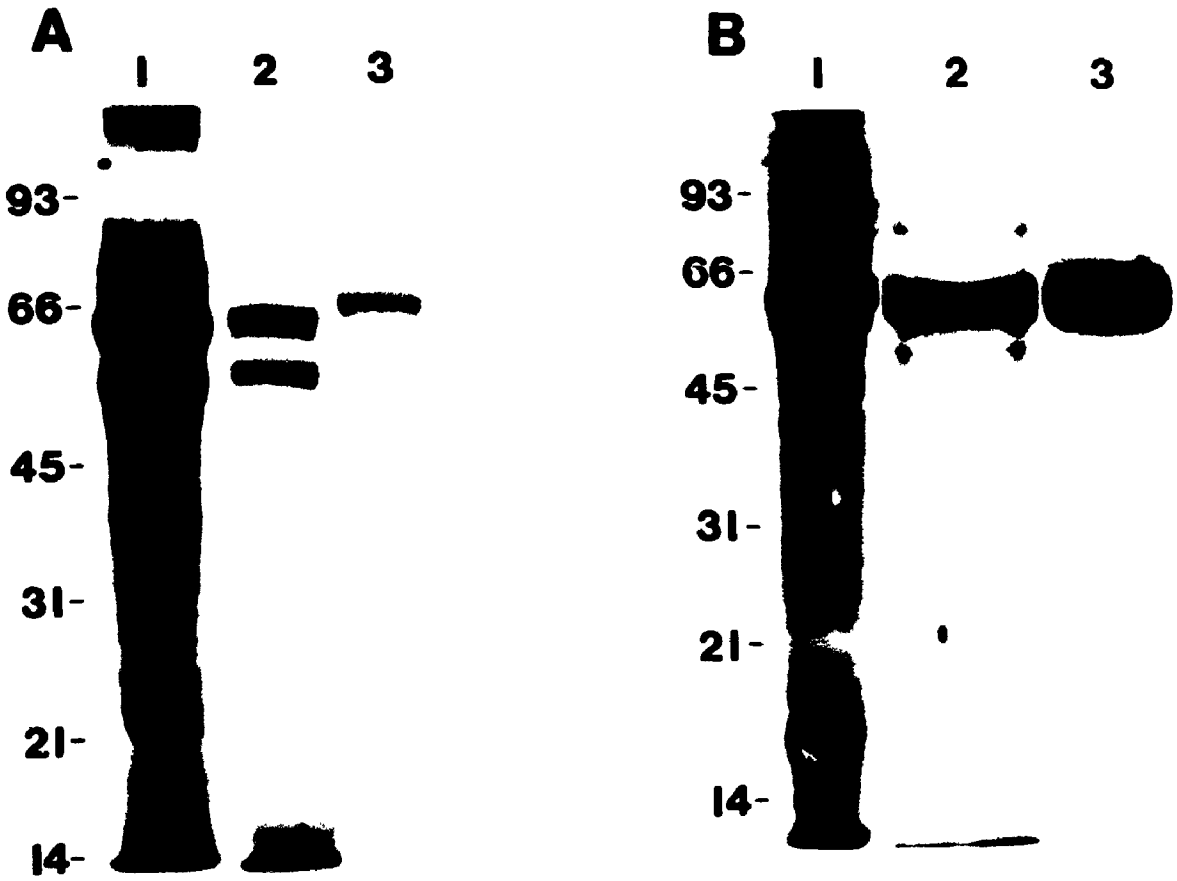
Although other methods of mfVSG purification have been published (Cardoso de Almeida *et al.*, 1984) perhaps the most expeditious and certainly the most familiar to our lab is the method developed by Clarke *et al.* (1984). The procedure consists of two steps: extraction of whole viable trypanosomes with 0.1% TFA followed by direct purification using reverse phase HPLC. VSG typically eluted as the largest peak at 30-35% organic solvent (isopropanol). VSG purity at each of the steps in the procedure was followed by SDS PAGE analysis (Figure

2.1A). In this example, WaTat 1.13 mfVSG was purified to near homogeneity. As can be seen in lane 1, the VSG (ca. 63 kDa) is the most predominant band in the starting material (whole cells). TFA extraction (lane 2) reduces the number of contaminants to bands of 53 kDa, 40 kDa and low molecular weight material <16 kDa. HPLC fractionation results in 90-95% pure mfVSG (lane 3) with the only detectable contaminant migrating to 60 kDa. Interestingly, the purified VSG exhibits significantly slower migration than the same VSG in the whole cell lysate. This suggests that its migration may be affected by other proteins during electrophoresis.

2.3.1.2 sVSG Purification by Osmotic Shock

The most efficient method available for purifying sVSG (Cross, 1975) was routinely employed for isolating WaTat VSGs. It also consists of a two step procedure involving an osmotic shock treatment which activates an endogenous glycan phosphatidylinositol-specific phospholipase C which cleaves the mfVSG at the membrane anchor releasing sVSG into the medium. After pelleting the "naked" trypanosome ghosts, the supernatant is applied to a DEAE cellulose column equilibrated with PBS pH 7.0 which retains virtually all contaminants leaving VSG in the flow through. SDS PAGE analysis of samples taken at each step in the procedure are shown in Figure 2.1B. Osmotic shock results in the release of highly enriched VSG (lane 2) which is further purified to homogeneity after application to DEAE cellulose (lane 3). Contaminants appear to be undetectable even with heavy loadings such as the one displayed.

Figure 2.1: SDS PAGE analysis of VSG purifications. A) Purification of mfVSG. Lane 1, trypanosome whole cell lysate (30 μg total protein); lane 2, TFA extract (2 μg); lane 3, RP-HPLC purified mfVSG (500 ng). B) Purification of sVSG. Lane 1, trypanosome whole cell (30 μg total protein); lane 2, osmotic shock supernatant (5 μg); lane 3, DEAE cellulose column flow through fraction (5 μg). Gels contained 10% acrylamide and WaTat 1.13 was used for both analyses. Proteins were visualized by staining with coomassie blue R-250. Positions of BioRad low molecular weight standards are indicated (in kDa) at the left of each gel.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31-
21-
14-
B
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14-

2.3.2 Relative Molecular Weights of WaTat VSGs

VSGs from the WaTat isoVAT can be distinguished from one another by their unique mobilities on SDS PAGE gels (Figure 2.2, lanes 1-4). Also included in the analysis is a non-isoVAT VSG designated WaTat 1.5 (lane 5). Relative molecular weight estimations for the WaTat VSGs examined here are: WaTat 1.1, 60 kDa; WaTat 1.12, 63 kDa; WaTat 1.13, 62 kDa; WaTat 1.14, 57 kDa and WaTat 1.5, 66 kDa. These estimates are within the range of molecular weights for VSGs reported by others (Turner, 1985).

2.3.3 Effects of Deglycosylation on Relative Molecular Weights of WaTat sVSGs

In order to insure that differences in M_r among isoVAT VSGs were not simply the result of differential glycosylation, sVSGs were deglycosylated using N-glycanase. According to the manufacturer (Genzyme), N-glycanase will cleave all classes of N-linked oligosaccharides directly at the N-linkage, thus removing the entire oligosaccharide. With the exception of the oligosaccharide moiety found in the glycolipid anchor, which is attached to the C-terminal amino acid via an amide linkage (Holder, 1983), all VSG oligosaccharides are N-linked (Holder and Cross, 1981).

SDS PAGE analysis of native and deglycosylated isoVAT VSGs is shown in Figure 2.3. Deglycosylation with N-glycanase results in a molecular weight shift to greater mobility for each of the VSGs. The observed molecular weight shifts are: WaTat 1.1, 3400 Da; WaTat 1.12, 3500 Da; WaTat 1.13, 3400 Da and WaTat 1.14, 1800 Da. Also of note is the observation that native WaTat 1.13 sVSG resolves into

Figure 2.2: Relative molecular weights of WaTat sVSGs. Lane 1, WaTat 1.1 sVSG; lane 2, WaTat 1.12 sVSG; lane 3, WaTat 1.13 sVSG; lane 4, WaTat 1.14 sVSG; lane 5, WaTat 1.5 sVSG. A 10-15% gradient gel was used for the analysis and 3 μg protein was loaded per lane. Bands were visualized by staining with coomassie blue R-250. Positions of BioRad low molecular weight standards are indicated (in kDa) at the left.


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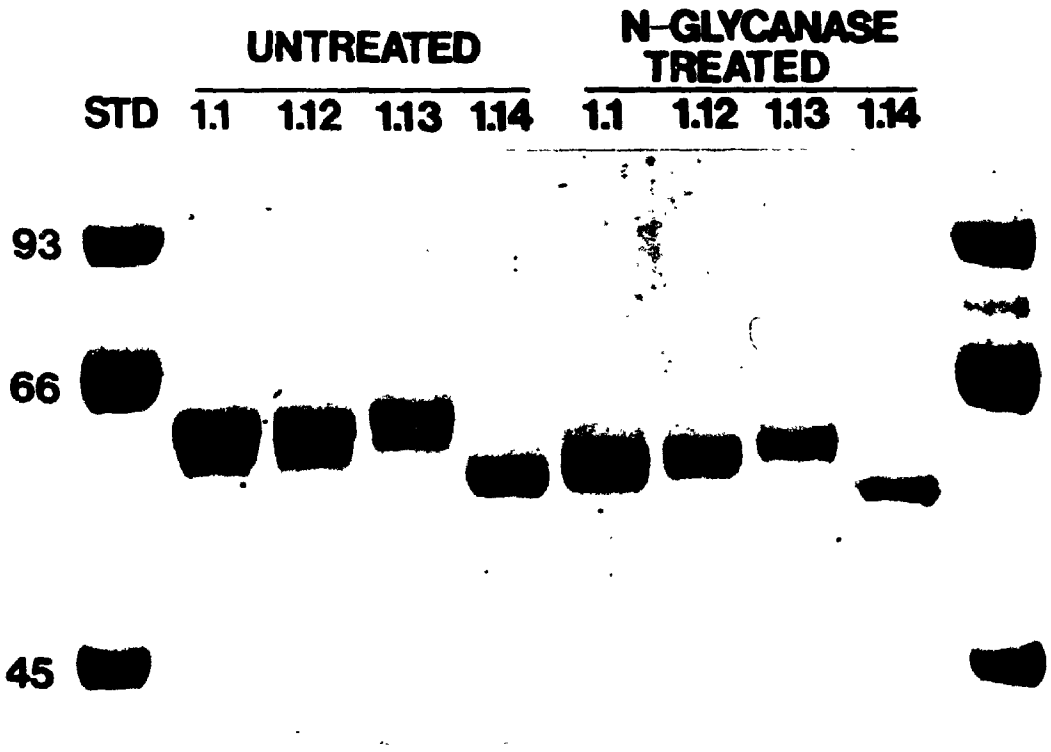
21-
14-

Figure 2.3: Deglycosylation of WaTat isoVAT VSGs with N-glycanase. 7.5% SDS PAGE analysis of untreated and N-glycanase treated isoVAT VSGs. Lane assignments are as indicated at the top of each lane. Deglycosylation was achieved using 10 units/ml N-glycanase in 0.1 M sodium phosphate buffer (pH 6.5) containing 10 mM EDTA, 0.1% Triton X-100, 0.5% SDS, and 0.1% 2-mercapto-ethanol at 37°C for 18 hours. STD, molecular weight standards (kDa). Bands were visualized by staining with coomassie blue R-250.


UNTREATED
N-GLYCANASE TREATED
STD
1.1
1.12
1.13
1.14
1.1
1.12
1.13
1.14
93
66
45

a doublet of bands at 62.1 kDa (major band) and 58.9 kDa (minor band) - a difference of 3200 Da. In fact, WaTat 1.13 consistently runs as a doublet on SDS PAGE gels of 10% polyacrylamide or less and the same phenomenon has been observed for at least one other VSG (WaTat 1.2 - G. Stroffolino, personal communication).

The degree of molecular weight shift observed upon treatment with N-glycanase correlates with the number of N-glycosylation sites predicted for each of the corresponding VSGs (Figure 4.13 for WaTat 1.12, data not shown for WaTats 1.1, 1.13, & 1.14). In this regard, variants 1.1, 1.12 and 1.13 appear to have two oligosaccharide moieties while WaTat 1.14 VSG only harbours one. This suggests that the minor band observed in the purified WaTat 1.13 sVSG preparation is the fully deglycosylated form.

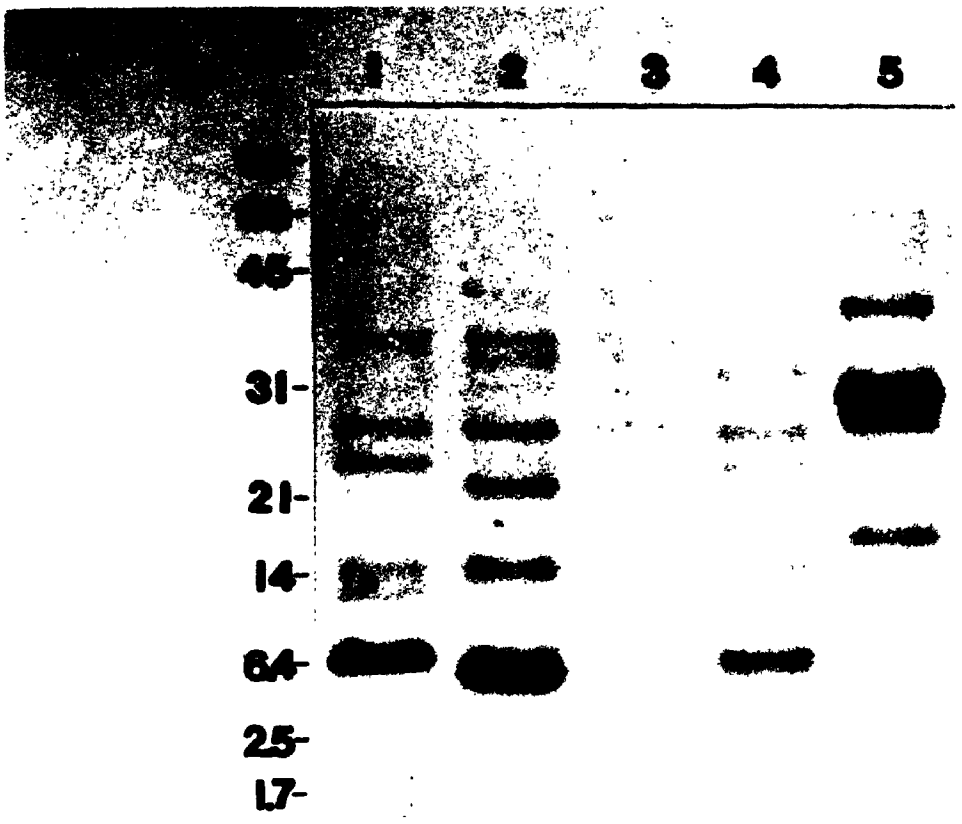
2.3.4 Peptide Mapping Experiments

2.3.4.1 CNBr Fragmentation of WaTat sVSGs

SDS PAGE resolved fragments generated by CNBr cleavage of WaTat isoVAT sVSGs show marked similarities (Figure 2.4A, lanes 1-4) while the pattern observed for the control VSG, WaTat 1.5 (lane 5), is entirely distinct. VSG fragments migrating to 27.5 kDa and 17.4 kDa are common to all four isoVAT VSGs while bands at 35.5, 25.7, 13.5 and 10.5 are common to three of the four VSGs. This leaves a total of only 4 bands which are unique to one VSG or another. In contrast, none of the fragments generated by CNBr cleavage of WaTat 1.5 sVSG co-migrate with fragments generated from isoVAT VSGs. This suggests that

Figure 2.4: Peptide mapping analysis. A) WaTat sVSG peptide maps generated with CNBr. Lane 1, WaTat 1.1; lane 2, WaTat 1.12; lane 3, WaTat 1.13; lane 4, WaTat 1.14 and lane 5, WaTat 1.5. 2 μg of each digest was loaded per lane. VSGs were reduced and carboxymethylated prior to cleavage with a 400 molar excess of CNBr in 70% formic acid at 20°C for 24 hours. B) WaTat sVSG peptide maps generated with SV8. Lane assignments are the same as for Figure 2.4A. VSGs were digested with SV8 at 1:20 (w/w) enzyme to substrate ratio in 1X SDS PAGE sample buffer at 20°C for 5 hours. 4 μg of each digest was loaded per lane. The arrow at the right of the SV8 gel indicates the position of a band present in the SV8 protease preparation.

Both analyses were performed on 10-15% Fling gradient gels as described in section 2.2.5.2. Positions and molecular weights (in kDa) of BioRad low molecular weight standards and CNBr cleaved sperm whale myoglobin are indicated at the left of each gel. Both gels were stained with coomassie blue R-250 in order to visualize peptides.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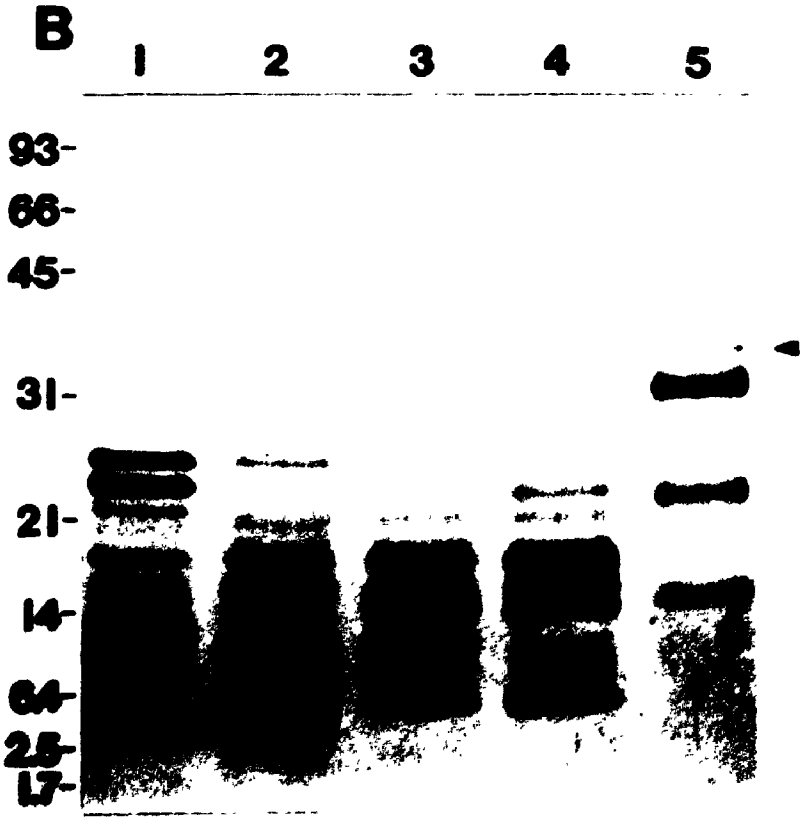
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significant structural homologies exist among the WaTat isoVAT VSGs particularly with respect to methionyl residues.

2.3.4.2 Staphylococcus V8 Protease Cleavage of WaTat sVSGs

SV8 protease is a very useful and highly specific enzyme which is capable of cleaving polypeptides at the N-terminal side of the acidic residues - aspartic acid and glutamic acid. SDS PAGE analysis of VSG fragments generated with SV8 protease are shown in Figure 2.4B. The patterns observed in this analysis are significantly more complex than those generated with CNBr and consequently isoVAT VSGs are more readily distinguished from one another. Nevertheless, obvious similarities exist between isoVAT VSG fragment patterns as compared to WaTat 1.5. The band (marked by arrow at right), which is common to all samples, is a component of the SV8 preparation. The results of this analysis are taken as further evidence for the existence of significant structural homologies among WaTat isoVAT VSGs.

2.4 Discussion

Information generated from investigations of VSG structure likely hold the key to understanding how the trypanosome uses this glycoprotein as an effective replaceable barrier, shielding underlying non-variant membrane proteins. Furthermore, results from these and other experiments form the basis upon which a solution to the VSG structure/function paradox can be found. Of critical importance to the latter assertion, is that structural information be generated for as many VSGs as possible, ultimately leading to complete delineation of their respective tertiary structures by crystallization and X-ray diffraction analysis. It follows then, that investigations of the structure of WaTat VSGs may form a significant contribution in this regard.

The results presented in this chapter involve partial delineation of structural characteristics and homologies existing among VSGs derived from trypanosomes sharing the same serotype. Each of these isoVAT VSGs was subjected to various biochemical tests and analyses including SDS PAGE, N-glycanase treatment and analytical peptide mapping. The results indicate that amino acid homologies are highly significant and appear to extend through the entire length of the sequence or a major portion thereof. Overall, the results presented here are consistent with the view that isoVAT VSGs are closely related but not identical.

A survey of all the results available on M_rs of *T. brucei* VSGs indicates that they can range from 52-69 kDa. Although, each of the WaTat VSGs examined in this study fall within this range, their sizes are not sufficiently consistent to be

indicative of structural relatedness.

Studies performed by others (Johnson and Cross, 1977) on several VSGs, established a range for carbohydrate composition of 7-17%. After correction for the fact that these estimates included the C-terminal glycan, which is not removed by N-glycanase, the results obtained in the present study are consistent with this established range. Of particular interest with respect to glycosylation of isoVAT VSGs is the observation that variant 1.14 VSG appears to have less carbohydrate than the others. The combined results from the present deglycosylation experiments and the number of N-linked glycosylation sites predicted from the now available full length sequences of isoVAT VSGs (Figure 4.13) suggest that WaTat 1.14 VSG has only one oligosaccharide moiety while the others have two. More specifically, all four VSGs appear to be glycosylated at amino acid position 266, but unlike the others, WaTat 1.14 is not glycosylated at position 401. It should be interesting to compare the effects of this difference on the regional tertiary structure of the polypeptide, once crystallization and X-ray diffraction studies, currently in progress, are complete.

As presented in this chapter, the results of analytical peptide mapping experiments using SV8 protease and CNBr to examine WaTat 1.1 and WaTat 1.12 VSGs, are consistent with the tryptic peptide mapping results previously presented for the same VSGs (Barbet *et al.*, 1982). However, the current study expands the analysis to include the WaTat 1.13 and 1.14 VSGs and further solidifies the close structural relationship that exists within the group. The now available full length

sequences determined from cDNAs (Appendix I) fully confirm the interpretation of the peptide mapping results. The observation that isoVAT VSGs are highly homologous is consistent with the idea that they are members of a gene family. Although the existence of VSG gene families has been implied from the results of southern analysis (Parsons *et al.*, 1983), none of these families have been characterized in great detail. Therefore, the WaTat isoVAT family is the first to be analyzed with respect to sequence and structure. This has interesting implications for the evolution of VSG genes. From the significant primary structure homology existing throughout the entire sequences of isoVAT VSGs, it would appear likely that this gene family was generated as a result of gene duplication events followed by sequence divergence. Judging from the relatively limited number of mutations, it seems likely that isoVAT VSG genes are still in the early stages of divergence. Although the role played by homologous recombination in VSG gene evolution, ie. through the creation of novel hybrid VSG genes, has been well documented (Pays *et al.*, 1983; Roth *et al.*, 1986; Longacre and Eisen, 1986; Thon *et al.*, 1990), VSG gene evolution propagated by point mutation has not previously been observed. This suggests that the trypanosome uses at least two mechanisms for generating novel VSG genes. The ramifications of VSG gene evolution are intimately linked to the function of the molecule as a polymorphic antigen, therefore they will be discussed in greater detail in chapter 3 which deals with the immunochemical properties of WaTat VSGs.

Now that WaTat isoVAT VSGs have been established as being closely

related, it will be interesting to observe the effects of apparently randomly distributed amino acid substitutions on VSG tertiary structure. Computer modelling should enable us to highlight deviations resulting from such substitutions. It may also be insightful to compare the interpretation of these studies with those generated from comparative tertiary structure analyses of non-related VSGs. Not only might this further resolve the inherent VSG structure/function paradox, it could also cast light on the elusive rules that govern the dependency of higher ordered structure on primary sequence.

Chapter 3 - Immunochemistry of WaTat isoVAT VSGs

3.1 Introduction

When VSG was discovered as the principal trypanosome antigen recognized by mammalian host immune response, most researchers were interested in characterizing neutralizing antibodies ie. antibodies capable of binding to surface-exposed antigenic determinants. In particular, the intent was to ultimately map the locations of neutralizing epitopes on the VSG. In eventual pursuit of this goal, Barbet and colleagues (Barbet *et al.*, 1982) prepared several monoclonal antibodies against both purified sVSG and lethally irradiated living cells of variant WaTat 1.1. In subsequent experiments (Clarke *et al.*, 1987), attempts were made to isolate VSG fragments containing epitopes recognized by neutralizing antibodies. These efforts failed because all of the neutralizing MAbs recognized non-linear antigenic determinants. In fact, though several laboratories have reported isolating neutralizing MAbs, no documented reports describing the structure of a neutralizing epitope have been published. Supporting the idea that neutralizing epitopes may all be non-linear, are the results of an experiment in which polyclonal antibodies prepared against VSG fragments, failed to bind to surface-exposed sites (Clarke *et al.*, 1987). This suggests that it may be very difficult or even impossible to produce neutralizing antibodies capable of recognizing linear epitopes on VSG.

At this point, immunochemical research regarding WaTat VSGs changed emphasis somewhat - from characterization of neutralizing epitopes to surveying

immunochemical structure. This survey was initially based on the specificity of MAbs created against WaTat 1.1 (Barbet *et al.*, 1982). Results indicated 6 out of 7 MAbs had the ability to recognize WaTat 1.12, in addition to WaTat 1.1. With this information available, I was given the mandate to further characterize the immunochemical properties of WaTat VSGs, particulary including the two newest additions to the family, WaTat 1.13 and WaTat 1.14. Among the questions remaining at the time, were: are surface-exposed epitopes completely conserved among isoVAT VSGs, can some measure of relatedness be deciphered using monoclonal antibodies and still in casual pursuit of the original goal, can monoclonal antibodies be prepared with specificity for linear neutralizing epitopes, given the results of Clarke *et al.* (1987)?

In order to answer the above questions, rabbit polyclonal antibodies were prepared against each WaTat sVSG and mouse MAbs were prepared against whole, denatured and fragmented WaTat isoVAT VSGs. Most MAbs were prepared against fragments because of particular interest in characterizing linear epitopes. In order to avoid the possibility of destroying potentially important epitopes, mice were immunized with overlapping fragments generated with SV8 protease and CNBr in separate digests. The following nomenclature system was created to name resulting MAbs: the first letter (or in one case, two letters) indicates the form of VSG used for immunization (V = whole VSG, P = peptides (fragments) and dn = denatured), the next two numbers indicate which VSG was used for immunization (12 = WaTat 1.12, etc.) and the last two characters indicate identity of the

hybridoma clone. The only MAbs, included in this study, not identified using this system are WAT 8A1 and WAT 20A1 and TRYP 22A1. These MAbs were prepared against pure VSG (WAT 8A1 or WAT 20A1) or whole trypanosomes (TRYP 22A1) of variant 1.1 (Barbet *et al.*, 1982).

Presented in this chapter are the results of studies designed to determine the immunochemical properties and antigenic similarities of VSGs belonging to the WaTat isoVAT. The results contained herein, suggest that, antigenically, isoVAT VSGs are highly similar but not identical. This is consistent with the results of structural analyses presented in chapter 2.

3.2 Materials and Methods

3.2.1 Chemicals and Biochemicals

All of the following laboratory chemicals were purchased as reagent or analytical grade or better. Tween 20, citric acid and nickel chloride were obtained from Baker while Sigma supplied the 2,2'-azino-bis (3-ethylbenz-thiazoline sulfonic acid) (ABTS) and diaminobenzidine (DAB). Freund's adjuvants, fish gelatin and hydrogen peroxide were purchased from Gibco, Norland and BDH respectively. Secondary antibodies were supplied by Jackson Immunoresearch, strep-tavidin/horse radish peroxidase (SA-HRP) complex was obtained from Amersham and Charles River provided the laboratory animals.

3.2.2 Preparation of Antisera

Rabbits (New Zealand white) were injected with 500 μg VSG in Freund's complete adjuvant (FCA) intramuscularly in two sites. Adjuvant was prepared by adding 400 μl of 1.25 mg/ml VSG in H2O to 600 μl of FCA in a 3cc disposable syringe. This mixture was then sonicated using a Fisher Scientific model 300 sonic dismembrator at 35% full power with 20 second pulses. Between pulses, the mixture was cooled on ice for approximately 30 seconds. The adjuvant was judged ready for injection when it became too viscous to move when the syringe was turned upside down. For secondary immunizations (booster), rabbits were injected 4 weeks later with 100 μg VSG prepared in Freund's incomplete adjuvant (FIA). The adjuvant was prepared in the same manner as described for the primary immunizations and injections were performed subcutaneously at four sites. Two

weeks following the secondary immunizations, rabbits were bled from the ear and the anti-VSG antibody response was tested in ELISA assays (section 3.2.4). For the full scale harvest of blood, cardiac puncture was performed using a 60 cc syringe fitted with an 18 gauge needle. Rabbits were anaesthetized with ether prior to exsanguination. The blood was allowed to clot overnight at 4°C in 50ml centrifuge tubes. Clots were then loosened with a wooden swab stick and the tubes were centrifuged at 500xg for 10 minutes at 4°C. The supernatant serum was collected, heat inactivated at 56°C for 30 minutes and stored at 4°C after adding sodium azide to 0.01% (w/v).

3.2.3 Preparation of Monoclonal Antibodies

6-8 week old balb/c mice (usually 4 animals per experiment) were initially immunized with 50 μg antigen in FCA followed by a secondary immunization with 10 μg antigen in FIA three weeks later. Adjuvants were prepared as described in section 2.2.5 and injections were performed intraperitoneally. 10-14 days following secondary immunization, mice were bled from the tail and sera were tested for anti-VSG antibodies by either dot blot assays or ELISAs (see sections 3.2.7 and 3.2.4 respectively). In each case the mouse exhibiting the highest anti-VSG titres was given a final boost of 10 μg antigen in sterile PBS, delivered by intravenous injection into the tail vein 3 days prior to fusion. Fusions were performed using a modification of the Kohler and Milstein (1975) procedure (Davis *et al.*, 1986). Hybridomas were screened by standard solid phase ELISA on both native VSG and VSG fragments generated by CNBr or SV8. Cleavages were performed as

described in section 3.2.4. Hybridomas secreting anti-VSG antibodies were cloned twice by limiting dilution. The antigen preparations used to generate each monoclonal antibody (MAb) were as follows. dn13A1 was derived from a mouse immunized with reduced and carboxymethylated ie. permanently denatured (Crestfield *et al.*, 1963) WaTat 1.13 VSG (cmVSG). WAT 8A1 and 20A1 MAbs were prepared against WaTat 1.1 sVSG, while V13C5 and V13B1 were made against WaTat 1.13 sVSG. A mixture of CNBr and SV8 fragments derived from WaTat 1.12 sVSG was used to generate MAbs P12A1, P12B1, P12C1, P12D1 and P12E1. Similarly, P13A2, P13B1 and P13C2 were generated from mice immunized with CNBr/SV8 fragments of WaTat 1.13 sVSG and P14A2 and P14B1 from WaTat 1.14 fragments.

3.2.4 ELISA Assays

ELISA plates (Nunc-Immuno Plates) were antigen coated by dispensing 100 µl/well antigen (100 ng/ml) in distilled H2O (ie. 10 ng antigen/well) and allowing the solvent to evaporate overnight in a 37°C non-humid incubator. Plates were then blocked for 0.5-1.0 hours with 3% (w/v) fish gelatin in PBS containing 0.05% (v/v) tween 20 (PBS-T). Plates were washed once with PBS-T then primary antibody (MAb or antiserum) was added and incubated for 1.0-1.5 hours. After 5 washes, secondary antibody (biotin labelled goat anti-rabbit IgG heavy and light chains or biotin labelled goat anti-mouse IgG and IgM heavy and light chains, diluted 1:2000) was added and also incubated 1.0-1.5 hours. After 5 further washes, SA-HRP (1:5000) was added and incubated 15 minutes. Following 3 washes with PBS-T

and 2 washes with PBS, a citrate buffer (50 mM citric acid pH 4.0) containing 200 μM ABTS and 0.015% (v/v) hydrogen peroxide was added and colour was allowed to develop for 15 minutes in the absence of light. Colour intensity was analyzed at 405 nm using a MK II Titertek Multiskan Plus ELISA plate reader. All immunochemical reagents, including the SA-HRP complex, were diluted in PBS-T containing 1% (w/v) fish gelatin and all washes were performed with PBS-T unless otherwise stated. Additionally, all incubations were performed at room temperature.

3.2.5 Immunofluorescence Assays

Indirect immunofluorescence assays (IFAs) on living trypanosomes were performed in order to assess the surface binding capabilities of monoclonal and polyclonal antibodies. Trypanosomes were grown in rats or mice and semi-purified by collecting the buffy coat after centrifugation of whole blood (see also section 2.2.3). Trypanosomes were adjusted to $2x10^6$/ml in PSG + 10% FBS and dispensed into 12x17 mm borosilicate glass test tubes (20 μl/tube) on ice. 50 μl of anti-VSG antibody (monoclonal or polyclonal) or control (conditioned myeloma growth medium or normal rabbit serum) were added neat or diluted in PSG/FBS and incubated for 1 hour at 0°C. Trypanosomes were washed 3 times by adding 1 ml ice cold PSG/FBS, followed by centrifugation at 400xg at 4°C for 5 minutes in an American Scientific Products Omnifuge RT with C1726-20 rotor. After the final wash, pellets were resuspended in 50 μl secondary antibody (fluorescein conjugated goat anti-mouse IgG or fluorescein conjugated goat anti-rabbit IgG), diluted 1:50 in PSG/FBS and incubated for 1 hour at 0°C. Trypanosomes were

again washed 3 times, then microscopically examined immediately using a Leitz Dialux 20 microscope fitted with a UV source attachment.

3.2.6 Deglycosylation of WaTat 1.13 VSG

Deglycosylation of WaTat 1.13 VSG, for the purpose of determining the specificity of monoclonal antibodies for carbohydrate or proteinaceous epitopes, was performed as described in chapter 2 section 2.2.6.

3.2.7 Dot Blots

Dot blots were performed according to the method described by Clarke *et al.* (1987). Briefly, 50 ng portions of antigen, each in 1 μl distilled H_2O, were spotted onto nitrocellulose filters (Schleicher and Schuell) in a grid pattern corresponding to the positions of wells in a 96 well microtitre plate. Filters were then blocked in PBS-T containing 3% (w/v) fish gelatin for 0.5-1.0 hours. Meanwhile, anti-VSG antibodies were diluted and placed in the wells of a 96 well microtitre plate previously blocked with fish gelatin. A home-made silicon rubber gasket was placed over the microtitre plate which was overlaid by the nitrocellulose filter. The microtitre plate lid fitted with a layer of spongy foam rubber was then placed on top and the entire apparatus clamped together with carpenter's clamps. The unit was then inverted such that the antibody solutions covered the nitrocellulose in each well and allowed to incubate for 1.0-1.5 hours. The apparatus was then disassembled and the filters washed 3 times in PBS-T for 15 minutes each. Filters were then placed in a solution of secondary antibody (biotin labelled goat anti-mouse IgM and IgG heavy and light chains or biotin

labelled goat anti-rabbit IgG heavy and light chains) diluted 1:2000, for 1.0-1.5 hours. Filters were again washed 3 times, as above then immersed in a solution of SA-HRP (1:5000). Following 3 10 minute washes in PBS-T, filters were briefly rinsed in PBS. Positive reactions were visualized by placing the filter in PBS containing 0.5 mg/ml diaminobenzidine (DAB), 0.3 mg/ml nickel chloride and 0.012% hydrogen peroxide. All incubations were performed at room temperature and PBS-T was used for the washes unless stated otherwise. Also, all immunological reagents were diluted in PBS-T containing 1.0% (w/v) fish gelatin.

3.3 Results

3.3.1 Immunofluorescence; Rabbit Polyclonal Antibodies

Although it was clear, based on their selection with an anti-WaTat 1.1 antiserum, that WaTat trypanosome variants 1.12, 1.13 and 1.14 shared common surface-exposed epitopes with WaTat 1.1, it was not certain whether common epitopes existed between all possible pairings of these variants. To this end, antisera versus each VSG was prepared, including one outside the isoVAT (WaTat 1.5) and tested in an indirect immunofluorescence assay on each variant. The results, summarized in Table 3.1, indicate complete cross-reactivity exists among isoVAT members and specificity is maintained, ie. no reactivity was observed with variant 1.5 except with its homologous antiserum. This suggests that each variant shares at least one common surface-exposed epitope with all other variants.

3.3.2 Immunofluorescence; Mouse Monoclonal Antibodies

To determine the specificity of our MAbs for VSG epitopes; surface-exposed in the context of the coat or buried because of adjacent VSG molecules (cryptic epitopes), indirect immunofluorescence assays were performed on living trypanosomes. Included as a positive control in the assays was MAb TRYP 22A1 (see Barbet *et al.*, 1982). No detectable surface fluorescence was observed for any of the MAbs tested, excluding the positive control (Table 3.2). Therefore, it can be concluded that all of these MAbs bind to cryptic antigenic determinants.

3.3.3 Effects of Deglycosylation on the Immunospecificity of Monoclonal Antibodies

Since VSGs are glycoproteins, it is important for the purpose of epitope

Table 3.1: Polyclonal antisera cross-reactivities with surface-exposed epitopes. Immunofluorescence assays were performed on living trypanosomes as described in section 3.2.5. Polyclonal antisera were prepared in rabbits against WaTat sVSGs as described in section 3.2.2. Relative fluorescence intensity is indicated by +, ++, or +++, from lowest to highest, by subjective determination. - indicates no fluorescence observed. NRS, normal rabbit serum. Modified from Carruthers and Clarke (1988).

Antiserum	Variant Antigenic Type 1.1	1.12	1.13	1.14	1.5
anti-1.1	+++	++	++	++	-
anti-1.12	+++	+++	++	+++	-
anti-1.13	+++	++	+++	++	-
anti-1.14	+++	++	++	+++	-
anti-1.5	-	-	-	-	++
NRS	-	-	-	-	-

Table 3.2: Monoclonal antibody specificities for surface-exposed epitopes. Immunofluorescence assays were performed on living trypanosomes as described in section 3.2.5. MAb TRYP 22A1 (or polyclonal antisera) was used as a positive control in the assays. MAbs were prepared as described in section 3.2.3. Fluorescence intensity is indicated by +, ++, or +++, from lowest to highest, by subjective determination. - indicates no fluorescence observed. NRS, normal rabbit serum. ND, not determined.

MAb	Variant Antigenic Type				
	1.1	1.12	1.13	1.14	1.5
Tryp 22A1	+++	-	-	+++	-
WAT 8A1	-	-	-	-	-
WAT 20A1	-	-	-	-	-
dn13A1	-	-	-	-	-
V13C5	-	-	-	-	-
P14A2	-	-	-	-	-
P14B1	-	-	-	-	-
P13A2	-	-	-	-	-
P13C2	-	-	-	-	-
P12A1	ND	-	ND	ND	ND
P12B1	ND	-	ND	ND	ND
P12C1	ND	-	ND	ND	ND
NMS	-	-	-	-	-

mapping to know the specificity of MAbs for protein or carbohydrate antigenic determinants. To obtain this information, MAbs were tested for binding to native and N-glycanase treated WaTat 1.13 sVSG in dot blot assays. As stated in chapter 2, N-glycanase removes all classes of N-linked oligosaccharides from glycoproteins by cleaving directly at the asparaginyl-oligosaccharide linkage. Observed is a 3400 Da shift down in M_r of native versus N-glycanase treated WaTat 1.13 (Figure 3.1A). All MAbs tested bound equally well to treated and untreated VSG, thus revealing specificity for antigenic determinants on the polypeptide (Figure 3.1B).

3.3.4 VAT Specificities of Monoclonal Antibodies

Although experiments with antisera demonstrated the existence of extensive cross-reactivity among isoVAT variants, the degree of cross-reactivity could not be ascertained, due to the polyclonal nature of the antibodies. On the other hand, MAbs, by virtue of their supreme specificity, are ideal for examining the extent of antigenic relatedness of isoVAT VSGs.

In order to determine our MAbs' binding specificities for members of the isoVAT group, standard ELISA and western blotting techniques were employed. WaTat 1.5 was included as an antigenically non-related control. MAbs were tested on native VSGs as well as SV8 and CNBr generated fragments. Results of these experiments, summarized in Figure 3.2, show that most MAbs exhibit "pan specificity", that is, they recognize all four members of the isoVAT. However, 5 out of 15 MAbs are "sub-pan specific" by virtue of their specificity for three or fewer

Figure 3.1: Monoclonal antibody specificity for deglycosylated sVSGs. A) 7.5% SDS PAGE analysis of native (-N-glycanase) and N-glycanase treated (+ N-glycanase) WaTat 1.13. Positions and sizes (in Daltons) of molecular weight markers (MW STD) are indicated on the left. Bands were visualized by staining in coomassie blue R-250. B) Dot blot assay testing MAb specificity for native (- N-glycanase) and N-glycanase treated (+ N-glycanase) WaTat 1.13. Treated and untreated VSG are from the same preparations analysed in Figure 3.1A. Assay was performed as described in section 3.2.7. NMS, normal rabbit serum. Modified from Carruthers and Clarke (1988).

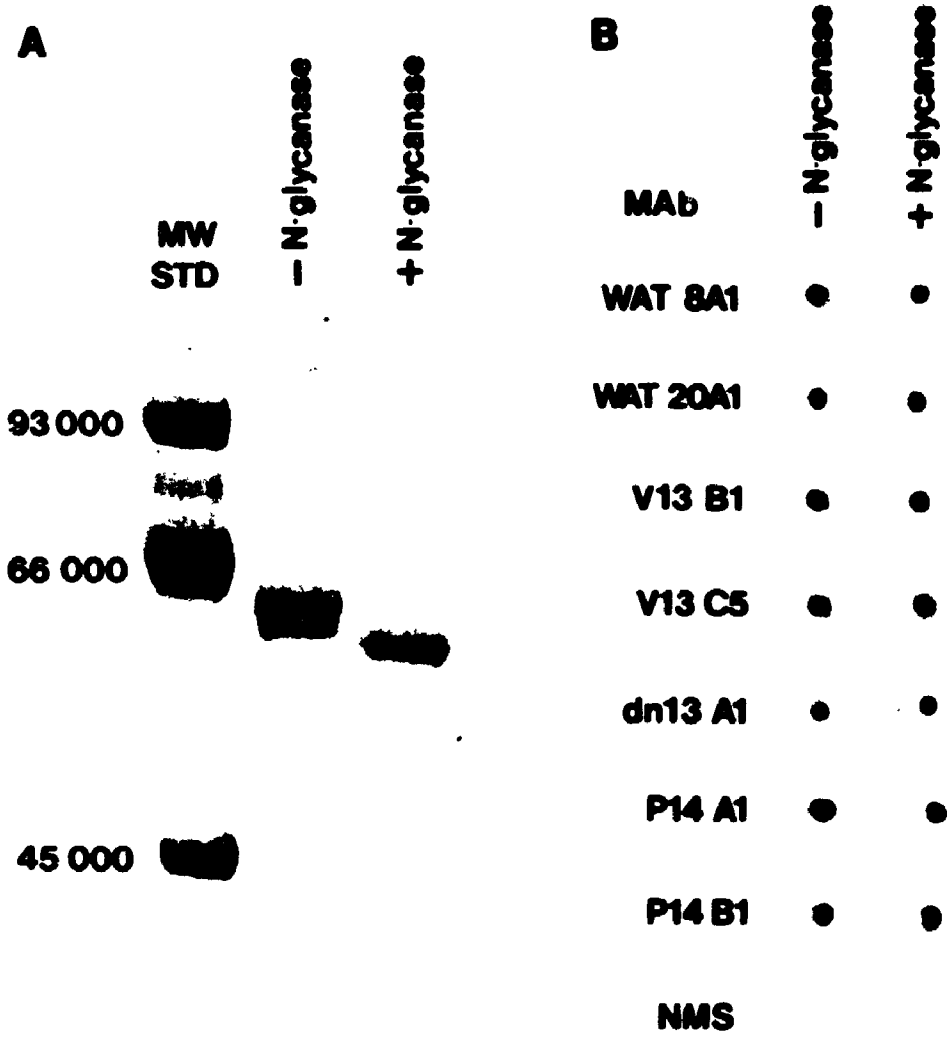

A
MW STD
- N-glycanase
+ N-glycanase
93 000
66 000
45 000
B
MAb
- N-glycanase
+ N-glycanase
WAT 8A1
WAT 20A1
V13 B1
V13 C5
dn13 A1
P14 A1
P14 B1
NMS

Figure 3.2: VAT specificities of monoclonal antibodies. Specificities were determined using standard ELISA and western blotting techniques on native VSG and SV8 protease and CNBr generated VSG peptides. Open boxes indicate positive recognition while closed boxes indicate lack of detectable binding. Specificity of MAbs for linear epitopes was determined by ability to bind to small molecular weight peptides (<10 kDa) in western blots.

Monoclonal Antibody	Group	Specificity					Linear Epitope
		1.1	1.2	1.3	1.4	1.5	
VI3BI	PAN SPECIFIC					■	YES
VI3C5						■	YES
PI3A2						■	YES
PI3BI						■	YES
PI3C2						■	YES
PI4A2						■	YES
PI4BI						■	YES
PI2AI						■	YES
WAT 8AI						■	YES
WAT 20AI						■	YES
PI2BI	SUB PAN SPECIFIC			■	■	■	YES
PI2CI				■	■	■	YES
VI2DI		■		■	■	■	YES
VI2EI		■		■	■	■	YES
dnI3AI		■	■		■	■	NO

members of the group. The ability of each MAb to recognize small M_r VSG fragments (<10 kDa) in western blots was considered evidence for specificity towards a linear epitope (first column on the right). In all cases specificity is maintained within the isoVAT as indicated by the absence of any detectable binding to WaTat 1.5.

Several of the pan specific MAbs have interesting properties worthy of description. WAT 8A1 and WAT 20A1 appear to recognize the same epitope, as indicated by their essentially identical recognition patterns on fragment western blots (Figure 4.1, section 4.3.1). In the same experiment it was observed that these MAbs do not recognized SV8 or CNBr generated WaTat 1.14 fragments. Interestingly, however, these MAbs will bind to intact native WaTat 1.14 VSG in ELISA assays. Although the basis of this discrepancy is not clear, in this case the positive ELISA result overrules the negative western blot results, hence the MAbs are considered to be pan specific. Another pan specific MAb, P13B1, is interesting because it appears to be fragment specific as evidenced by its complete inability to bind to native VSG in ELISA assay. Presumably the antigenic determinant it recognizes is not solvent accessible on the native VSG and fragmentation or perhaps denaturation of the VSG is required to expose its binding site.

With respect to the sub-pan specific MAbs, three (V12D1, V12E1 and dn13A1) demonstrate monospecificity. MAb dn13A1 is also interesting because, though it was prepared against permanently denatured VSG, it failed to bind to small M_r fragments in western blots and therefore it likely recognizes a non-linear

epitope.

Taken together, MAb specificity results suggest isoVAT VSGs are antigenically close relatives but not identical since sub-pan specific epitopes are detectable.

Results presented in chapter 2 and in other studies (Olafson *et al.*, 1984) suggest that WaTat isoVAT VSGs share common structural features at the amino acid sequence level. These findings were recently confirmed by sequencing full length cDNAs of isoVAT VSGs (appendix I). As mentioned in chapter 2, the sequences display point mutation substitutions at only 12% of the positions in the first 400 amino acids. In light of this data, it can be suggested that pan specific MAbs bind to completely conserved sequences or to sequences containing mutations that do not affect the integrity of the antigenic determinant. Conversely, sub-pan specific MAbs presumably bind to regions containing mutations that have an effect on binding specificity. The only way to definitively confirm this presumption was to determine the precise location and structure of antigenic sites recognized by these MAbs, as described in chapter 4.

3.4 Discussion

Findings presented in this chapter provide insight into the immunochemical characteristics of related VSGs from WaTat variants. Results were generated by examining antigenic determinants on VSGs using polyclonal and monoclonal antibodies. These tests were designed to coarsely demarcate determinants (immunofluorescence and deglycosylation experiments) and explore the distribution of sites among all four VSGs (ELISA). Results indicate: (a) surface-exposed epitopes are conserved on isoVAT VSGs; (b) most, but not all, antigenic determinants are shared by isoVAT VSGs and (c) none of the MAbs recognizing linear epitopes bind to neutralizing epitopes, however, they do recognize determinants on the polypeptide.

Recently, independent results (Barbet *et al.*, 1989) from immuno-fluorescence assays using antisera raised against each of the WaTat VSGs, confirmed the results of the same experiment described here. In both cases reciprocal cross-reactivity between all possible pairings of isoVAT variants was demonstrated. This suggests that isoVAT VSGs have the capacity to evoke similar B-cell responses, indicating shared immunogenic properties in addition to common antigenic properties.

The same report cited above (Barbet *et al.*, 1989) also describes the original anti-WaTat 1.1 MAbs (TRYP and WAT series) in terms of their specificities for WaTat 1.13 and WaTat 1.14 - properties not determined in previous studies (Barbet *et al.*, 1982). Combining the results of these studies and present results;

of the 13 distinct (ie. distinguished from all others by competition assays or relative or definitive localization studies, see also chapter 4) antigenic determinants recognized by MAbs, 7 are pan-specific and 6 are sub-pan specific. In other words, a roughly equal proportion of pan vs sub-pan specific sites exist on isoVAT VSGs. Combining this observation with the results of sequence analysis of isoVAT VSGs, 88% identity in sequence results in approximately 50% identity in antigenic properties. This information is valuable because it demonstrates the dependency of immunochemical properties on primary sequence. Coupled with information of a similar nature from other families of antigens, it is conceivable that this relationship could be fully realized, resulting in a basis upon which one property could be predicted from the other. Given the rate at which protein sequences are being generated, such information may be of practical use for inferring the degree of antigenic similarity existing among the protein products of other gene families.

Results presented in this chapter further demonstrate the suitability of VSG as a model system for examining the effects of amino acid sequence variation on antigenic specificity. Since substitutions occurring within isoVAT VSG sequences have been revealed, definitive localization of pan and sub-pan specific antigenic determinants should at least partially disclose the nature of allowable (ie. in terms of maintaining specificity) and non-allowable replacements within antigenic sites.

As discussed in chapter 2 and further sustained by the evidence presented here, isoVAT VSGs are probably members of a gene family. During the course of a natural infection, expression of one member likely precludes expression of others

because immune memory results in rapid elimination of trypanosomes expressing cross-reactive serotypes (Cross, 1975). Therefore, VSG gene families may be of no immediate functional value to the parasite. However, they appear to be the products of an evolutionary process designed to rejuvenate and create novel VAT repertoires. This newly described mechanism is in addition to the established strategy of creating hybrid VSG genes by homologous recombination (Pays *et al.*, 1983; Roth *et al.*, 1986; Longacre and Eisen, 1986; Thon *et al.*, 1990).

As demonstrated by immunofluorescence assays on living trypanosomes, none of the MAbs used in the present study bind to surface-exposed antigenic determinants. This further strengthens the hypothesis that no linear epitopes exist on the exposed portion of the VSG while in the surface coat. Is this indicative of unique folding characteristics in the surface-exposed area? The recently solved VSG crystal structure (Freymann *et al.*, 1990) demonstrates the presence of random structure segments forming loops across the top of the molecule. It remains unclear why this folding pattern is so poorly conducive to characterization with MAbs. Perhaps computer modelling of VSG oligomers or experiments using antibodies prepared against synthetic peptides corresponding to exposed regions, will resolve this issue.

Although it has been assumed that the area of VSG exposed on the trypanosome surface is at the top of the molecule, experimental evidence regarding the degree of exposure remains unavailable. For this reason and as discussed above, for further analysis of the VSG model, I have developed and

applied an epitope mapping system to precisely demarcate the location and structure of antigenic sites bound by anti-VSG MAbs. Results of these efforts are presented in the following chapters.

Chapter 4 - Localization of Epitopes on WaTat isoVAT VSGs

4.1 Introduction

As mentioned in chapter 1, two types of antigenic determinants exist on the VSG as it is arranged in the surface coat: cryptic and surface-exposed. The latter type can be further divided into two sub-types, neutralizing and non-neutralizing (Masterson *et al.*, 1988), depending on the ability of antibodies directed against them to protect against infection. No surface-exposed determinants have been precisely demarcated thus far because of their extreme lability which is suggestive of non-linear character. The idea that cryptic and surface-exposed epitopes do not overlap has been interpreted from the results of topological analyses (*via* competition assays) of antigenic determinants (Hall and Esser, 1984; Miller *et al.*, 1984a). However, Masterson *et al.* (1988) postulate that MAbs able to bind to the surface of living trypanosomes yet unable to neutralize infectivity are actually binding to partially exposed sites. More experimentation is apparently required to fully understand the relationship between surface exposure and neutralizing ability.

Only two reports are available regarding partial characterization of linear antigenic sites on VSGs. Miller *et al.*, (1984b) were able to locate determinants on large CNBr and tryptic fragments. Results indicated that all determinants examined were in the N-terminal domain of the VSG. Furthermore, the only neutralizing MAbs used recognized a 19 kDa N-terminal fragment. However, these neutralizing sites were non-linear and further resolution was unattainable. In another study (Davis and Bennett, 1982), six distinct linear epitopes were partially characterized using

a method similar to the fragment western technique used in this thesis. Although the authors suggested that all six sites were accessible on the trypanosome surface, immunofluorescence assays were performed using fixed cells, therefore, it remains to be proven that these sites are truly surface-exposed on the viable parasite. Moreover, no results regarding definitive localization of determinants were described.

When discussing the structure of antigenic determinants, elements of higher ordered structure must be considered. As mentioned, most epitopes on proteins are non-linear ie. comprised of separate regions of the polypeptide brought together by folding. Clearly, therefore, integrity of non-linear determinants is highly reliant on tertiary structure. In contrast, linear epitopes are less dependent on tertiary structure because they are each confined to a single region of the polypeptide. Nevertheless, linear antigenic sites can be significantly contingent on secondary structure. For example, if antibody binds to an alpha helical region, it can only interact with one face of the helix. Furthermore, if the longitudinal dimension of a paratope is approximately 30 Angstroms (Amit *et al.*, 1986), then it could potentially interact with 4-5 turns of an alpha helix (5.75 Angstroms/turn, Wilson *et al.*, 1984). Therefore, contact residues at each end of the determinant could be separated by as many as 16 residues. On the other hand, contact residues in an antigenic site present on a random structured segment can be contiguous and as little as 5-7 residues in length (Bittle *et al.*, 1982; Strohmaier *et al.*, 1982). These examples serve to depict how secondary structure can

significantly influence the length of sequence constituting a linear epitope. This is one consideration not taken into account by strategies involving characterization of linear epitopes exclusively to short sequences (5-7 residues) using synthetic peptides (Houghten *et al.*, 1986; Geysen *et al.*, 1987b; Geysen *et al.*, 1987a; Geysen, 1985) or other means (Scott and Smith, 1990).

Presented in this chapter are the results of epitope mapping studies revealing the relative distribution of antigenic sites on VSG (relative localization), in addition to delimitation of linear determinants on the antigen (definitive localization). Seven distinct linear antigenic determinants have been mapped to regions of 6-45 amino acids in length by deletion analysis. A discussion of the effects of amino acid substitutions on antigenic specificity is included. In addition, relevance of epitope positions to VSG structure and molecular organization in the surface coat is considered.

4.2 Materials and Methods

4.2.1 Chemicals and Biochemicals

All chemicals were purchased from commercial sources and were reagent or analytical grade or better. BSA and one-phor-all™ buffer for restriction digests and all DNA enzymes, including polymerases, ligase, phosphatase and restriction endonucleases, were products of Pharmacia. BDH was the supplier for sodium chloride, sodium hydroxide, chloroform, isoamyl alcohol, sucrose, ethidium bromide, boric acid, magnesium chloride, glucose, calcium chloride and formamide, while glycerol, manganese chloride and ammonium persulfate were obtained from Fisher. Pierce, Mallinckrodt and Fluka were the suppliers for cyanogen bromide, potassium acetate and hexamine cobalt chloride respectively. Sodium Bicarbonate, sodium carbonate, ethylenediamine tetracetate (EDTA), phenol, bromophenol blue, potassium chloride, acetic acid, ammonium acetate and urea were supplied by Baker. Yeast extract, casamino acids and agar were products of Difco, while Boehringer Mannheim provided dithiothreitol (DTT), staphylococcus V8 protease (SV8), ribonuclease A (RNAse), deoxyribonuclease (DNAse), agarose, isopropyl-Beta-D-thiogalactopyranoside (IPTG), deoxy- and dideoxynucleotide triphosphates (d & ddNTPs) and Tris. Polyethylene glycol, acrylamide, sodium dodecyl sulfate (SDS), xylene cyanol, coomassie blue R-250 and N,N,N',N',-tetramethylethylene diamine (TEMED) were obtained from Serva. Caldon supplied dimethylsulfoxide (DMSO), Aldrich provided rubidium chloride and the alpha-phosphorothiate nucleotides were produced by Promega. Finally, Sigma

was the source of ampicillin (amp), lysozyme, proline, thiamine, dimethyldichloro silane and gamma-(methylacryloxy)-propyltrimethoxy silane (Bindsilane™).

4.2.2 Fragment Western Blots

WaTat isoVAT VSGs were cleaved with CNBr or SV8 according to the methods described in sections 2.2.7.1 and 2.2.7.2 respectively. Fragments were resolved on 12-17% linear gradient gels (1 μg/lane) as described in section 2.2.5.2. Fragments were "renatured" and electrophoretically transferred to immobilon-P (Millipore) membranes according to Dunn (1986). Briefly, after PAGE, gels were incubated in 50mM Tris pH 7.4 + 20% (v/v) glycerol (renaturation buffer) for 1 hour at room temperature. Immobilon-P membranes were prepared for use according to the manufacturers instructions. Fragments were then electrophoretically transferred at 30 volts for 18-20 hours, in 10 mM sodium bicarbonate, 3 mM sodium carbonate, pH 9.9, 20% (v/v) methanol, using a BioRad mini-protean II transfer unit. Following transfer, antigenic fragments were detected in the same manner as for dot blots (see section 3.2.7).

4.2.3 Routine DNA Manipulation Procedures

4.2.3.1 Plasmid Isolation

4.2.3.1.1 Rapid Boil Method

When using restriction enzymes to examine insert sizes of deletion mutants, an adaptation of the Holmes and Quigley (1981) rapid boil method was used (Sambrook *et al.*, 1989).

4.2.3.1.2 Alkaline Lysis Method

A modified version of the Birnboim and Doly (1979) procedure (Kraft *et al.*, 1988) was used when performing medium scale plasmid isolation (eg. preparation for exo III deletions). The only major alteration Kraft *et al.* (1988) make of the Birnboim and Doly method (1979) was to add a PEG precipitation step at the end of the procedure.

4.2.3.2 Restriction Enzyme Digests

Restriction enzyme digests were carried out at DNA concentrations of 0.1 μg/ml in one-phor-all™ buffer (1X or 2X according to the manufacturers recommendations) containing 100 μg/ml bovine serum albumin at an enzyme to substrate ratio of 2-4 units/μg. All digests were performed at 37°C for 1-2 hours unless otherwise noted and enzymes were heat inactivated at 65°C or 85°C (depending on the enzyme) for 20 minutes. In instances where particular restriction enzymes could not be heat inactivated, samples were phenol/chloroform extracted, then ethanol precipitated (see section 4.2.4), before reconstitution and further processing.

4.2.3.3 Phenol/Chloroform Extraction and Ethanol Precipitation of DNA

Phenol (ACS standards grade) was redistilled and stored in 70 ml aliquots at -20°C. Prior to use, phenol was equilibrated in 100 mM tris pH 8.0 by repeated mixing with an equal volume of buffer until the observed pH reached 7.8 or higher. This equilibrated phenol solution was stored at 4°C for up to 6 months. For DNA extractions, phenol was mixed in equal proportion with a 24:1 chloroform:isoamyl alcohol solution and then re-equilibrated by adding an equal volume of 100 mM

tris pH 8.0 and vortexing. The aqueous phase was aspirated after brief centrifugation and the phenol/chloroform solution was then mixed with the DNA sample. Following centrifugation, the aqueous phase, containing the DNA, was removed.

Ethanol precipitations were routinely performed by adding 0.04 volumes 5 M NaCl and 2 volumes -20°C ethanol followed by incubation at 0°C for 10 minutes. Precipitated DNA was recovered by centrifugation at 10,000xg for 10 minutes at 4°C. Pellets were generally washed once with -20°C 70% (v/v) ethanol, recentrifuged, then dried under vacuum.

4.2.3.4 Gel Electrophoresis of DNA

Samples were prepared for electrophoresis by adding 1/6th volume of DNA gel loading buffer [0.25% (w/w) bromophenol blue, 40% (w/v) sucrose in H_2O]. Size standards included either or both Hind III or Pst I cut lambda DNA. Size standards were heated for 2 minutes at 70°C immediately prior to loading. In most cases electrophoresis was continued until the bromophenol blue dye front reached the bottom of the gel. After staining in ethidium bromide (approx. 200 ng/ml in H_2O) for 5-10 minutes, bands were visualized using an Ultra-Violet Products, Inc transilluminator.

4.2.3.4.1 Agarose Gel Electrophoresis of DNA

0.8-2.0% (depending on the DNA size) agarose gels were used for routine examination of plasmids and medium to large DNA fragments (greater than 300 base pairs). Electrophoresis was carried out in Tris-acetate buffer (TAE, 40 mM

Tris-acetate, 1 mM EDTA) using a mini-sub DNA cell or wide mini-sub cell, both manufactured by BioRad. The gel dimensions were 10.0x6.5 cm (length by width) for the mini-sub cell and 10.0x15.0 cm (length by width) for the wide mini-sub cell.

4.2.3.4.2 Polyacrylamide Gel Electrophoresis of DNA

When resolution of small DNA fragments was necessary, 4-12% (depending on the fragment sizes) polyacrylamide gels were used. Gels were electrophoresed in Tris-borate buffer (TBE, 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) at 100 volts, until the bromophenol blue dye front reached the bottom of the gel. Gel apparatuses used included a BioRad mini-protean II and a model V-16 vertical gel electrophoresis unit from Bethesda Research Laboratories (BRL). The BRL unit, being the larger of the two, was used when analyzing large numbers of samples and/or when maximum resolution was required. Gel dimensions were 17.8x21.0 cm (length by width) for the BRL unit and 7.0x8.5 (length by width) for the BioRad apparatus.

4.2.3.5 DNA Ligations

DNA ligations were most frequently carried out at 15°C in one-phor-all™ buffer with ATP added to 1 mM. Blunt end ligations were performed using 5 units of T4 ligase for 15-18 hours while 1 unit T4 ligase and a 2 hour incubation was used for sticky end ligations. In cases where recircularization was desired, DNA concentration was adjusted to 100 μg/ml. Insert/vector ligations were routinely conducted at an insert to vector ratio of 1:1 and DNA concentration of 10 μg/ml.

In situations where undesired recircularization of the vector (ie without an insert) was possible, the vector was dephosphorylated with alkaline phosphatase prior to ligation. Dephosphorylations were carried out with 0.5 units of alkaline phosphatase in 1X one-phor-all™ buffer at 37°C for 30 minutes, then heat inactivated at 85°C for 15 minutes.

4.2.4 Transformations

Transformation of *Escherichia coli* strains JM109, mGP1-2 and JM109 (DE3) with plasmids (and recombinant constructs thereof) pUC18, pT7-7 and pGEMEX-1 respectively, was accomplished using either the Hanahan (1983) method, or by electroporation using a BioRad Gene Pulser unit according to the manufacturers directions. Note: in situations when expression was not desired, pGEMEX constructs were used to transform JM109 instead of JM109 (DE3).

4.2.5 cDNA Sub-Cloning

All VSG cDNAs used in these experiments were kindly provided by Dr A.F Barbet (U. of Florida).

4.2.5.1 WaTat 1.14 cDNA Insertion into pUC18

Full length cDNA encoding the WaTat 1.14 VSG was inserted into the Pst I site in the multiple cloning region of pGEM4 using the technique of GC tailing (Deng and Wu, 1981, performed in Dr A.F. Barbet's lab, U. of Florida). GC tailing leaves the insert with flanks of GC sequences in the order of 20 to 60 bases in length. The cDNA was excised from pGEM4 using Eco RI and Hind III and ligated into pUC18 cut with the same restriction enzymes (NB. the multiple cloning regions

of pGEM4 and pUC18 are virtually identical). Both the insert and double cut pUC18 were purified by cutting the respective bands out of a 0.8% agarose gel. Fragments were recovered from the agarose using a prep-a-gene kit according to the manufacturers directions (BioRad). The 5' GC tail, untranslated region and leader peptide sequence were then removed after double cutting the plasmid with Kpn I and Bam HI, followed by exonuclease III deletions (section 4.2.6). Patch screens (section 4.2.5) using monoclonal and polyclonal antibodies were performed in order to select clones expressing 1.14 VSG. Positive clones were then examined on western blots to confirm expression. Plasmid DNA from these clones was sequenced (section 4.2.9) to identify those which still contained the entire coding region of the mature VSG. Following selection of a clone almost completely devoid of 5' leader sequence, a Hind III/Bam HI fragment corresponding to bases 33-377 of the plasmid pBR322 was inserted into the multiple cloning region HindIII site at the 3' end of the VSG cDNA insert. This fragment contains translational stop codons in all three reading frames within a 30 base region. It was necessary to include this "pBR322 stops" fragment because when deletions are made in the 3' to 5' direction, the VSG's natural translational termination codon is lost. Transformants generated from the construct containing the pBR322 stops fragment were again screened on western blots to confirm continued expression. This construct was given the designation p14.

4.2.5.2 WaTat 1.13 cDNA Insertion into pT7-7

The WaTat 1.13 cDNA was inserted into the expression plasmid pT7-7 (a

kind gift of Dr Stanley Tabour, Harvard University) in the same manner as the 1.14 cDNA was sub-cloned into pUC18 (section 4.2.3.1). Subsequent steps leading to the isolation of full length clone expressing 1.13 VSG were also performed as described in section 4.2.3.1. In this case, however, the pBR322 stops fragment was not inserted because VSG expression directed from pT7-7 was sufficiently unstable to warrant termination of the experiment.

4.2.5.3 WaTat 1.12 and 1.14 cDNA Insertion into pGEMEX-1

An appropriate sticky end cloning procedure could not be devised for inserting the 1.12 and 1.14 cDNAs into pGEMEX-1, therefore a blunt end procedure was employed. Briefly, cDNAs for the 1.12 and 1.14 VSGs were excised from pGEM4 using Bam HI and Hind III and the vector was linearized with Apa I. cDNA fragments and the vector were then purified by agarose (0.8% w/v) gel electrophoresis and their ends made blunt with 1 unit of T4 DNA polymerase in 1X one-phor-all™ buffer containing 100 μM dNTP's. The fill-in reaction was allowed to proceed at 12°C for 15 minutes, followed by heat inactivation of the polymerase at 75°C for 10 minutes. cDNA inserts were then ligated into dephosphorylated vector (see also section 4.2.3.5) and used to transform JM109. Plasmid DNA was isolated from 9 transformants for each cDNA and screened for the presence of inserts using restriction enzymes. Plasmids harbouring inserts were then further analyzed by restriction enzymes in order to determine insert orientation. Clones in the correct orientation were subjected to deletion of the 5' GC tail, untranslated region and signal sequence using exonuclease III (section 4.2.5). The resulting

deletion mutants were screened on patch blots, then on western blots. Clones expressing VSG were then examined with respect to their insert sizes and for each VSG, one clone containing the entire sequence of its respective mature VSG was selected.

4.2.6 Exonuclease III Deletions

Deletion clones, expressing full length VSG or used in the epitope mapping experiments, were generated using exo III and an Erase-a-base kit according to the manufacturers instructions (Promega). Briefly, plasmids (10-12 μg) were first double cut with restriction enzymes (at the 5' or 3' end of the insert, depending on which direction deletions were to be made) such that a 3' overhang existed on the vector side and a 5' overhang or blunt end remained on the insert side. In situations where only a 5' overhang could be generated on the vector side, this site was cut first, protected by filling in with DNA polymerase I Klenow fragment and alpha-phosphorothioate nucleotides (dNTP analogues) and then cut with the second restriction enzyme. Following restriction, samples were phenol/chloroform extracted, chloroform extracted and ethanol precipitated, before reconstituting in exonuclease III buffer (66 mM Tris pH 8.0, 6.6 mM $MgCl_2$). After heating to 33°C and adding exo III, aliquots were removed at regular intervals (usually 10 or 15 seconds) and digested with S1 nuclease. Ends were repaired with klenow, then deletion plasmids were recircularized with T4 ligase and used to transform JM109, JM109 (DE3) or mGP1-2 depending on the vector.

4.2.7 Patch Screens

Initial screens for expression of either full length VSG or deletion products fc epitope mapping were performed using patch blots. Following transformation and growth overnight, colonies were picked and patched (inoculated onto a small area approx. 3x3 mm) in replicate on 1 M9 + supplements (50 μg/ml ampicillin, 1 mM thiamine, 20 μg/ml proline and 100 mM glucose) and 1 or more LB + ampicillin plates, depending on how many antibodies were to be used in the screen. The M9 plate was incubated at 37°C for 2 days then stored at 4°C for up to 6 months. Clones were stored on M9 media in order to minimize background expression and therefore avoid loss of expression due to toxicity of the VSG product. LB + amp plates were incubated for 5-6 hours until patches were clearly visible, then they were placed at 4°C for 1 hour. Patches were then lifted onto 0.2 μm nitrocellulose discs (millipore) and inoculated discs placed, patch side up, on fresh LB + amp and IPTG (1 mM) plates and incubated at 37°C overnight. Patches were lysed by placing discs on 3 mm Whatman filter paper soaked in 2% (w/w) SDS for 15 minutes. Discs were then exposed to chloroform vapour for 15 minutes and placed in a solution of 3% (w/v) fish gelatin in PBS-T containing 5 mg/ml DNAse and 50 mg/ml lysozyme for 30 minutes. The solution was then changed to 3% (w/v) gelatin minus DNAse and lysozyme for 30 minutes, after which cellular debris was rubbed off by hand. Blots were subsequently developed in the same manner as dot blots (section 3.2.7).

4.2.8 Analysis of Deletion Clones on Western Blots

Deletion clones expressing truncated VSG products were prepared for

western blotting as follows. 1.0 ml cultures in LB + amp were grown overnight at 37°C, then expression was induced by adding 1.0 ml LB + amp and IPTG (2 mM) and incubating for a further 3 hours at 37°C. For pUC clones (p14), cells were pelleted from 100 μl of each sample and reconstituted in 100 μl SDS PAGE sample buffer at 100°C and maintained at 100°C for 5 minutes. For pT7-7 and pGEMEX clones, 10 μl of each sample was pelleted and reconstituted in 100 μl sample buffer at 100°C and boiled for 5 minutes. Samples were centrifuged at 14,000xg for 5 minutes prior to loading. Samples were electrophoresed on 12% SDS PAGE gels (10 μl/lane), then semi-dry transferred to immobilon-P membranes using an American Bionetics model SBD-1000 Polyblot Transfer system according to the manufacturers instructions. Following electrotransfer, membranes were processed and developed as described for dot blots (section 3.2.7).

4.2.9 Peptide Mapping for the Identification of Expressed Recombinant VSG (Cleveland Mapping)

In order to confirm the identity of recombinant VSG expressed in *E. coli*, peptide mapping using SV8 protease was performed (Cleveland *et al.*, 1977). Briefly, WaTat 1.14 sVSG (8 ng and 24 ng) and lysates (prepared as described in section 4.2.7) of recombinant clones expressing 1.14 VSG from p14 (3.3 μl and 10 μl) and pGEMEX.14 (1.65 μl and 2.5 μl) were electrophoresed on 12% SDS PAGE gels. Following electrophoresis, gels were coomassie stained for 30 minutes and destained until the background was clear (see also section 2.2.5.1 for staining and destaining procedures). The gel was then placed on a light box and the VSG band

from each lane was excised using a scalpel blade. Gel slices were equilibrated in 5 ml of equilibration buffer (0.125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS and 0.1 M EDTA), then inserted into wells of a 10-15% Fling (Fling and Gregerson, 1986) gradient SDS PAGE gel (see also section 2.2.5.2). Samples were arranged into two groups designated "whole" (lighter loading) and "digest" (heavier loading). "Digest" samples were each overlaid with 5 μl SDS PAGE sample buffer containing 50 μg/ml SV8 protease, while "whole" samples were overlaid with sample buffer alone. Electrophoresis was initiated at 50 volts, until the bromophenol blue dye front reached the top of the separating gel, then the power was turned off and samples were allowed to digest for 30 minutes. Electrophoresis was continued at 100 volts until the dye front ran off the bottom of the gel (approx. 1.5 hours). Polypeptides were transferred (semi-dry) to immobilon membranes (see also section 4.2.7) and probed with monoclonal antibody P14B1 and developed according to the method described in section 3.2.7.

4.2.10 DNA Sequencing

Plasmid DNA from deletion clones defining the boundaries of an epitope (ie. either the smallest positive deletion clone or the largest negative deletion clone for each border) was sequenced using T7 DNA polymerase and dideoxy nucleotides according to a modified version of the protocol developed by Sanger (1977). Sequencing was performed in one direction only since deletion junction positions could be confirmed by comparing deletion clone sequences with previously deduced full length sequences of isoVAT VSGs (Appendix I). 2-5 μg of each

plasmid template (purified using the alkaline lysis method, section 4.2.3.1.2) in 20 μl of TE buffer was denatured by the addition of 2 μl of a 2 M NaOH/2 mM EDTA solution and incubation at 70°C for 5 minutes. 6 μl of 7 M ammonium acetate was added and templates were precipitated with 2 volumes of ethanol (-20°C, 60 minutes). Templates were pelleted at 14,000xg for 15 minutes at 4°C, washed once with 70% (v/v) ethanol and dried under vacuum. Samples were redissolved in 10 μl of sequencing buffer (20 mM magnesium chloride, 50 mM sodium chloride and 20 mM Tris pH 7.5) containing 10 ng of primer. Template/primer annealing was accomplished by heating the samples to 70°C for 5 minutes then allowing them to cool slowly to 30°C over approx. 30 minutes. Labelling reactions were performed by adding 1 μl labelling mix (7.5 μM dGTP, dCTP and dTTP in 40 mM Tris pH 7.5, 50 mM sodium chloride), 0.5 μl S^{35} dATP, 1 μl 100 mM DTT, 0.25 μl (1 unit) T7 polymerase and 2.75 μl H_2O and incubating at room temperature for 2-5 minutes. 3.5 μl of this mixture was transferred to each extension tube containing one of the four dideoxy nucleotides. All extension tubes contained 2.5 μl of nucleotide mix including 0.95 mM dNTPs in sequencing buffer. In addition, the A, G and T extension tubes each contained 14 μM ddATP, ddGTP and ddTTP respectively while the C tube contained 17 μM ddCTP. The extension reactions were carried out at 42°C for 10-30 minutes then 2.5 μl of stop solution (95% (v/v) formamide, 20 mM EDTA, 0.5% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol FF) was added and samples were denatured at 70°C for 5 minutes.

Primers used for sequencing deletion clones were synthesized, with the trityl

groups attached, using an Applied Biosystems Inc. (ABI) model 380A DNA synthesizer and purified on ABI OPC™ columns according to the manufacturers directions. After purification, primers were stored in aliquots at -70°C. The following primer, corresponding to bases 313-327 of pBR322 (translational stops region), was synthesized and used for sequencing the 3' junctions of pUC.14 deletion clones from the 3'-5' deletion series:

$^{5'}$TGCTCGCTTGGCTACT$^{3'}$.

In addition, for sequencing the 5' junctions of pGEMEX.14 and pGEMEX.12 from the 5'-3' deletion series', the following oligonucleotide primer which is complementary to bases 131-141 of pGEMEX-1 was prepared:

$^{5'}$CGCTGGTGGTGCTGGTACCG$^{3'}$.

Finally, a third oligonucleotide primer was synthesized for sequencing the 3' junctions of pGEMEX.14 from the 3'-5' deletion series. This primer corresponds to bases 3829-3845 of pGEMEX-1 and has the following sequence:

$^{5'}$TTACGCCAGGTTATCCG$^{3'}$.

In preparation for use, gel plates were soaked in 0.05 M sodium hydroxide,

washed with 5% (w/v) SDS, then rinsed with distilled water and dried. They were then rinsed with ethanol and dried again. The large plate was siliconized with 10 ml 5% (w/v) dimethyldichlorosilane in chloroform, then rinsed with ethanol. The small plate was treated with 5 ml of ethanol containing 17.5 μl gamma-tetracryloxy-propyl-trimethylsilane and 175 μl 10% acetic acid, then rinsed again with ethanol.

Gel mixes contained 7 M urea and 6% acrylamide in 1X TBE buffer and polymerization was catalyzed by the addition of 20 μl of TEMED (N,N,N',N'-tetramethylethylene diamine) and 150 μl 10% (w/v) ammonium persulfate (freshly prepared). After pouring, gels were allowed to polymerize overnight. A 30-60 minute pre-run was performed at 2000 volts in 1X TBE in order to heat the apparatus to the running temperature. 2.5 μl of each reaction mixture was loaded into wells formed using a shark's tooth comb and electrophoresis was carried out at 2000 volts for 2-5 hours, depending on the region of sequence to be read. After electrophoresis, the plates were separated, leaving the gel bound to the small plate. The gel was then fixed in 10% acetic acid for 20-30 minutes then dried directly on the small plate, at 70°C for 2 hours. Finally, the gel was exposed to X-ray film (Dupont) until the bands could easily be distinguished (usually 1-2 days depending on the specific activity of the S^{35} dATP).

4.2.11 Solution Phase Antibody Inhibition Studies Using Homologous VSG

Antibody inhibition assays using homologous VSG were conducted in order to examine the accessibility of epitopes on solution phase VSG. Before inhibition assays could be performed, it was necessary to generate titration curves for each

of the monoclonal antibodies in order to determine 50% $O.D._{MAX}$ points. This is defined as the dilution of antibody which produces half the maximum O.D. value and is also the point at which the greatest slope occurs in the titration curve. Titration curves were generated as follows. Plates were coated with antigen by dispensing 100 μl aliquots of WaTat 1.12 VSG at 100 ng/ml in distilled water into each well and allowing the water to evaporate at 37°C overnight. Plates were then blocked with 3% (w/v) fish gelatin (150 μl/well) in PBS-T for 1 hour. After 2 washes with PBS-T, 1/5 serial dilutions (100 μl/well), starting at neat for tissue culture supernatants or 1/1000 for ascites, of each monoclonal antibody were made directly in the plate. Each series was performed in triplicate using 1.0% (w/v) gelatin in PBS-T as the diluent. As negative controls, 3 wells per plate received diluent alone. Plates were incubated at 4°C for 1.5 hours. Further processing of the ELISA plates followed the procedure described in section 3.2.4. Titration curves were drawn based on average values from the triplicate series' and 50% $O.D._{MAX}$s were calculated.

Antibody inhibition assays were performed as follows. First, ELISA plates were blocked with fish gelatin as described above. After two washes with PBS-T, monoclonal antibodies, diluted to their respective 50% $O.D._{MAX}$ concentrations, were added at 100 μl/well. A dilution series of WaTat 1.12 sVSG, ranging from 0 ng/μl to 450 ng/μl in 50 ng/μl increments, was prepared and 1 μl from each dilution was added to respective wells containing monoclonal antibody. Inhibition series were also performed in triplicate, using wells that did not receive primary

antibody as negative controls. Plates were incubated at 4°C overnight followed by transfer to new, antigen coated and blocked plates. Plates were then incubated at 4°C for 1.5 hours and again developed following the procedure described in section 3.2.4.

4.3 Results

4.3.1 Relative Localization of Antigenic sites; Epitope Mapping *Via* Fragment Western Blots

The term "epitope mapping", in the broadest sense, can be used to describe any experiment that generates information regarding the relative distribution of antigenic sites. Traditionally, the most popular method of epitope mapping has involved the use of a competitive binding assay using an ELISA or radioimmunoassay (RIA) format (eg. Cecilia *et al.*, 1988; Wanidworananun *et al.*, 1989; Hufert *et al.*, 1989). This assay is based on the ability of an unlabelled MAb to inhibit the binding of a labelled MAb to the antigen. If two MAbs demonstrate competitive binding, then it is most often concluded that the epitopes to which they bind are either the same or juxtaposed. One advantage of this assay is that it can be used to examine both linear and non-linear epitopes. Although a topologic map can be generated from the results of competition assays, there is considerable uncertainty as to the conclusions that can be made regarding the proximity of epitopes. For this reason I decided to attempt to map epitopes by probing fragment western blots with MAbs and comparing the resulting immunoreactive profiles. The premise for this is: MAbs recognizing the same epitope will bind to common fragments resolved by SDS PAGE, thus producing identical or very similar banding patterns.

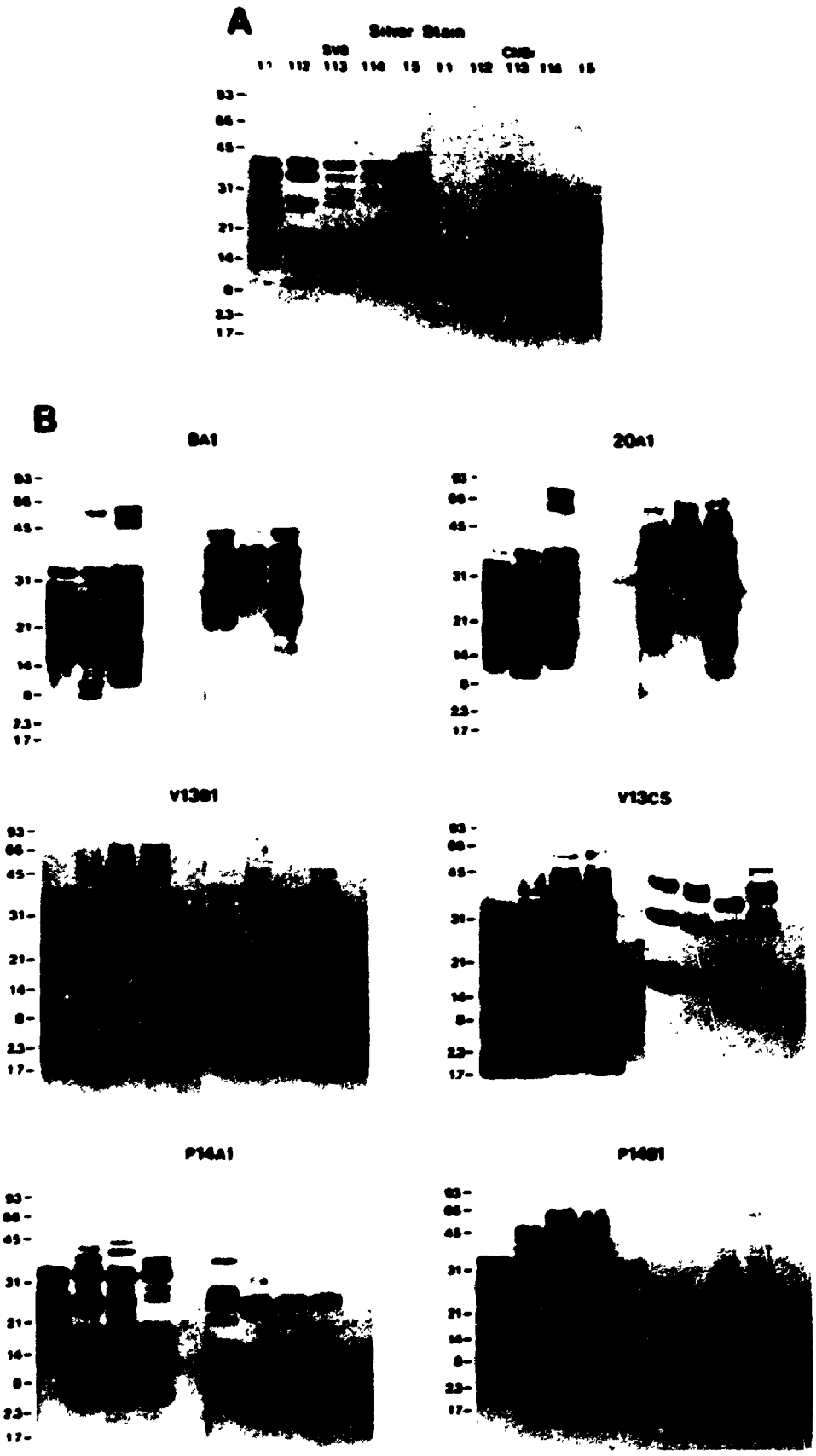
Results of epitope mapping experiments using fragment western blots are presented in Figures 4.1 and 4.2. They are presented separately because the

experiments were performed at different times, with different batches of peptide digests. Since all MAbs were not available at the same time, this discontinuity was unfortunately difficult to avoid. Nevertheless, comparisons can be made among MAbs within each experiment.

Basic fragment patterns revealed by silver staining are displayed in Figure 4.1A. The presence of high M_r fragments suggests these digests have not gone to completion. Fragment western blots (with the same lane assignments as in Figure 4.1A) probed with MAbs WAT 8A1, WAT 20A1, V13B1, V13C5, P14A1 and P14B1 are presented in Figure 4.1B. Fragment patterns generated by probing with MAbs WAT 8A1 and WAT 20A1 are virtually identical, suggesting that they bind to the same site or adjacent positions. Similarly, V13B1 and V13C5 display identical patterns, indicating they likely bind to a common epitope. Further evidence supporting this suggestion is unavailable due to the loss of V13B1 (see also section 3.3.4).

Distinct patterns are observed when fragments are probed with MAbs P14A1 and P14B1. This suggests that epitopes recognized by WAT 8A1/WAT 20A1, V13B1/V13C5, P14A1 and P14B1 are distinct from one another. Interestingly, however, epitopes for P14A1 and P14B1 have been localized to the same 25 amino acid region (section 4.3.2.4). This may provide a measurement of the resolution obtainable using the fragment western technique. With further regard to the P14B1 blot, two observations are noteworthy. First, immunoreactive fragments are observed in the negative control lane (WaTat 1.5). This appears to be the

Figure 4.1: Relative localization of antigenic sites; peptide western blots I. A) Silver stained gel of peptides generated with SV8 and CNBr. Lane assignments and cleavage reagents are as indicated at the top of the gel. B) Western blots probed with anti VSG MAbs. Lane assignments are the same as for Figure 4.1A and MAbs used as probes are indicated at the top of each blot. All gels are 10-15% Fling gradient gels, prepared and electrophoresed as described in section 2.2.5.2. Western blots were developed as described in section 4.2.2. Positions of a mixture of BioRad low molecular weight standards and CNBr cleaved sperm whale myoglobin are indicated (in kDa) at the left of each gel or blot. 8A1 = WAT 8A1; 20A1 = WAT 20A1.

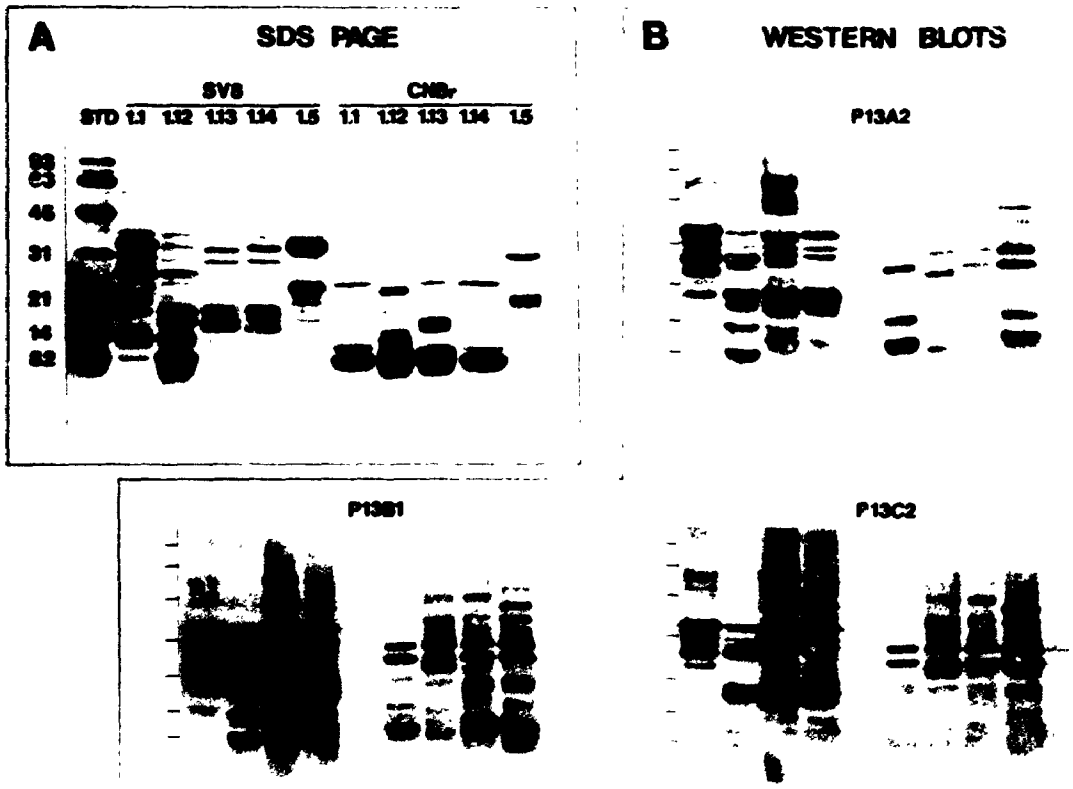

A
Silver Stain
SV8
CMB
11 112 113 114 15 11 112 113 114 15
93-
66-
45-
31-
21-
14-
8-
23-
17-
B
8A1
20A1
V13B1
V13C5
P14A1
P14B1

result of carry over of material from the adjacent lane (WaTat 1.14). Second, note the absence of detectable binding to CNBr cleaved WaTat 1.12 VSG. This observation was also confirmed by ELISA. One explanation, is that the WaTaT 1.12 VSG contains a unique additional methionyl residue which is somehow involved with the P14B1 epitope, such that when it is cleaved, the antigenic site is destroyed. Examination of isoVAT VSG sequences (Appendix I) reveals the presence of a unique methionine at position 57 of the WaTat 1.12 mature polypeptide. However, it is not clear how cleavage at this site would affect binding of P14B1 to its epitope, located somewhere in the first 25 amino acids (see also section 4.3.2.4).

Fragment westerns probed with P13A2, P13B1 and P13C2 (Figure 4.2B) exhibit some similarities in the middle and upper molecular weight ranges. However, in the lower molecular weight range distinct patterns are observed when blots probed with P13A2 and P13C2 are compared. This suggests the antigenic sites they recognize may be very close together but not identical. Definitive localization studies (section 4.3.2) have now shown these epitopes to be separated by 42 amino acids. The recognition pattern for P13B1 displays marked similarities to patterns generated with P13A2 and P13C2. This could suggest that the antigenic site to which it binds is positioned between the P13A2 and P13C2 epitopes. This, however, is completely unsubstantiated because the P13B1 epitope has not been definitively located.

Comparisons between the results of relative localization (fragment westerns)

Figure 4.2: Relative localization of antigenic determinants; peptide western blots II. A) SDS PAGE analysis of peptides generated with SV8 or CNBr, stained with silver. Lane assignments and cleavage reagents are as indicated at the top of the gel. Positions of molecular weight standards (STD, in kDa) are indicated at the left of the gel. B) Peptide western blots probed with anti-VSG MAbs. Indicated at the top of each blot are the MAbs used as probes. Lines illustrated at the left of each blot indicate the positions of molecular weight standards in the same order as for Figure 4.2A. All gels are 10-15% Fling gels (Fling and Gregerson, 1986), prepared and electrophoresed as described in section 2.2.5. Western blots were developed as described in section 4.2.2.


A
SDS PAGE
SV8
CNBr
STD 1.1 1.12 1.13 1.14 1.5 1.1 1.12 1.13 1.14 1.5
93
67
46
31
21
14
B
WESTERN BLOTS
P13A2
P13B1
P13C2

and definitive localization (deletion analysis) are valuable in providing a basis upon which conclusions can be made for the much more expeditious fragment western method. To this end, it appears that, for two MAbs: (1) identical recognition patterns likely indicate binding to the same or adjacent sites; (2) similar recognition patterns are indicative of proximal sites; but (3) distinct patterns may not always indicate non-propinquity between sites. Clearly, however, further testing is required in order to refine and solidify these rules.

4.3.2 Definitive Localization of Antigenic Sites: Deletion Analysis

In recent years, the use of synthetic peptides has been the most popular method for determining the definitive locations of epitopes (eg. Hazarika and Dedman, 1988; Isola *et al.*, 1989; Ohlin *et al.*, 1989). However, the cost of performing a comprehensive experiment using synthetic peptides to precisely locate epitopes is prohibitive for most laboratories. In attempting to devise an alternative strategy, I and, independently, others (Gill *et al.*, 1988) developed a deletion analysis scheme based on expression and detection of full length antigen and truncated products thereof. The premise for this strategy is if progressive deletions through a cDNA or gene of a given antigen are made and expressed as deletion clones they can be probed in succession with a MAb and at some point through the series the epitope recognized by the antibody will be deleted, resulting in absence of reactivity from that point onward. By performing the deletion analysis in both directions, one can define both boundaries of a given linear epitope. Perhaps the most efficient way to perform the analysis is to create 3' to 5'

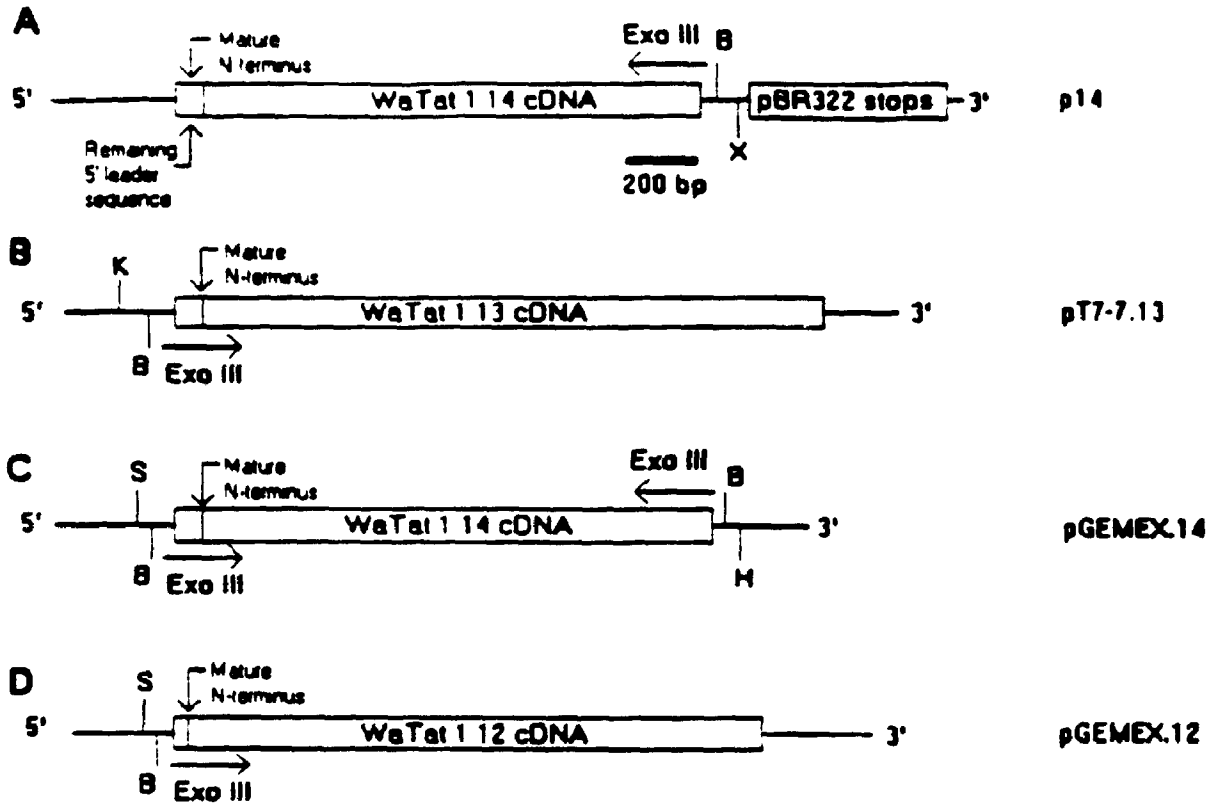
deletions first, isolate 10 or so deletion clones spanning the entire cDNA ("low resolution clones"), screen these with each MAb to determine approximate locations, then isolate "high resolution" clones which span the region of interest to precisely map the 3' or C-terminal-most boundary of the epitope. 3' to 5' deletions should be screened first because no reading frame disruption occurs. High resolution deletion clones can be immediately isolated from the 5' to 3' deletion series since epitope locations are already known.

Results generated from applying this strategy to localization of antigenic determinants recognized by anti-VSG MAbs are presented in this section.

4.3.2.1 Preparation of VSG cDNA Constructs and Expression of Full Length VSGs

Initial efforts to express VSG in *E. coli* involved insertion of the WaTat 1.14 cDNA into pUC18 (Figure 4.3A). pUC18 is an expression vector in which a multiple cloning site (MCS) is situated immediately downstream from the lac promoter and the first four codons of the lac Z gene. Foreign DNA sequences can be cloned into the MCS and expressed as fusion proteins, provided correct reading frame is maintained. In order to isolate an in frame VSG cDNA clone in pUC18, small 5' deletions were generated using exonuclease III. An in frame clone deleted to 10 codons prior to the mature N-terminus, as determined by DNA sequencing, was isolated by screening for expression with MAbs. A 345 bp restriction fragment from pBR322 containing stop codons in all three reading frames was then inserted at the 3' end of the cDNA. This construct was given the designation p14. Expression of full length recombinant WaTat 1.14 VSG (rVSG) from this construct was

Figure 4.3: Maps of cDNA constructs. A) WaTat 1.14 cDNA inserted into pUC18, construct designated: p14. B) WaTat 1.13 cDNA in pT7-7, designated pT7-7.13. C) WaTat 1.14 cDNA insert in pGEMEX-1, pGEMEX.14. D) WaTat 1.12 cDNA in pGEMEX-1, pGEMEX.12. Direction of exonuclease III (exo III) deletions is indicated on each construct. Restriction sites used for deletions are also indicated. B, Bam HI; X, Xba I; K, Kpn I; S, Sac I; H, Hind III. Except in the regions immediately surrrounding restriction sites, all illustrations are drawn to the scale displayed in A. Constructs were prepared as described in section 4.2.7.


A
Mature
N-terminus
Exo III
B
5'
WaTat 1 14 cDNA
pBR322 stops
3'
p14
Remaining
5' leader
sequence
X
200 bp
B
K
Mature
N-terminus
5'
WaTat 1 13 cDNA
3'
pT7-7.13
B
Exo III
C
S
Mature
N-terminus
Exo III
B
5'
WaTat 1 14 cDNA
3'
pGEMEX.14
B
Exo III
H
D
S
Mature
N-terminus
5'
WaTat 1 12 cDNA
3'
pGEMEX.12
B
Exo III

detectable on western blots using MAb P14B1 (Figure 4.4, lane 2). The band detected on western blots was confirmed to be 1.14 VSG by comparing the immunoreactive SV8 protease digest patterns (Cleveland maps, Cleveland *et al.*, 1977) of purified WaTat 1.14 sVSG and the expression product (Figure 4.4, lane 4 vs lane 5). Patterns for pure sVSG and rVSG are essentially identical except for an upward shift of ca. 2-4 kDa exhibited by the rVSG fragments. This shift can be accounted for by leader and tail sequences present on rVSG but absent from the sVSG.

Although valuable information regarding epitope locations was generated from deletion analysis of p14, less than optimal expression levels were a problem. For this reason I attempted to find another, more suitable, expression vector. Consequently, Stan Tabour (Harvard University) kindly provided plasmid, pT7-7, which can express foreign DNA sequences using a T7 polymerase based system induced by temperature shift (Tabour and Richardson, 1985). WaTat 1.13 cDNA was inserted into the MCS of pT7-7 (construct designated pT7-7.13, Figure 4.3B) and an in frame expression clone was isolated in the same manner as described above for WaTat 1.14 cDNA. Although expression levels were significantly greater than for p14 (Figure 4.5A), expression was unstable, as evidenced by the appearance of lower molecular weight bands after storage of the clone for 3 days (Figure 4.5B). It appears likely that expression of VSG product was mildly toxic to the host. For this reason, experiments with the pT7-7/1.13 construct were aborted.

The search for a better expression system ended when satisfactory results

Figure 4.4: Expression of full length VSG and identity confirmation by Cleveland mapping (Cleveland *et al.*, 1977). 10-15% SDS PAGE gel western blot analysis of VSG bands excised from a 12% gel of purified WaTat 1.14 sVSG and lysates of p14 and pGEMEX.14, untreated (lanes 1-3 respectively) or treated with SV8 protease (lanes 4-6 respectively), as described in section 4.2.12. Blot was probed with MAb P14B1. Positions of BioRad low molecular weight markers (in kDa) are indicated at the left of the blot.

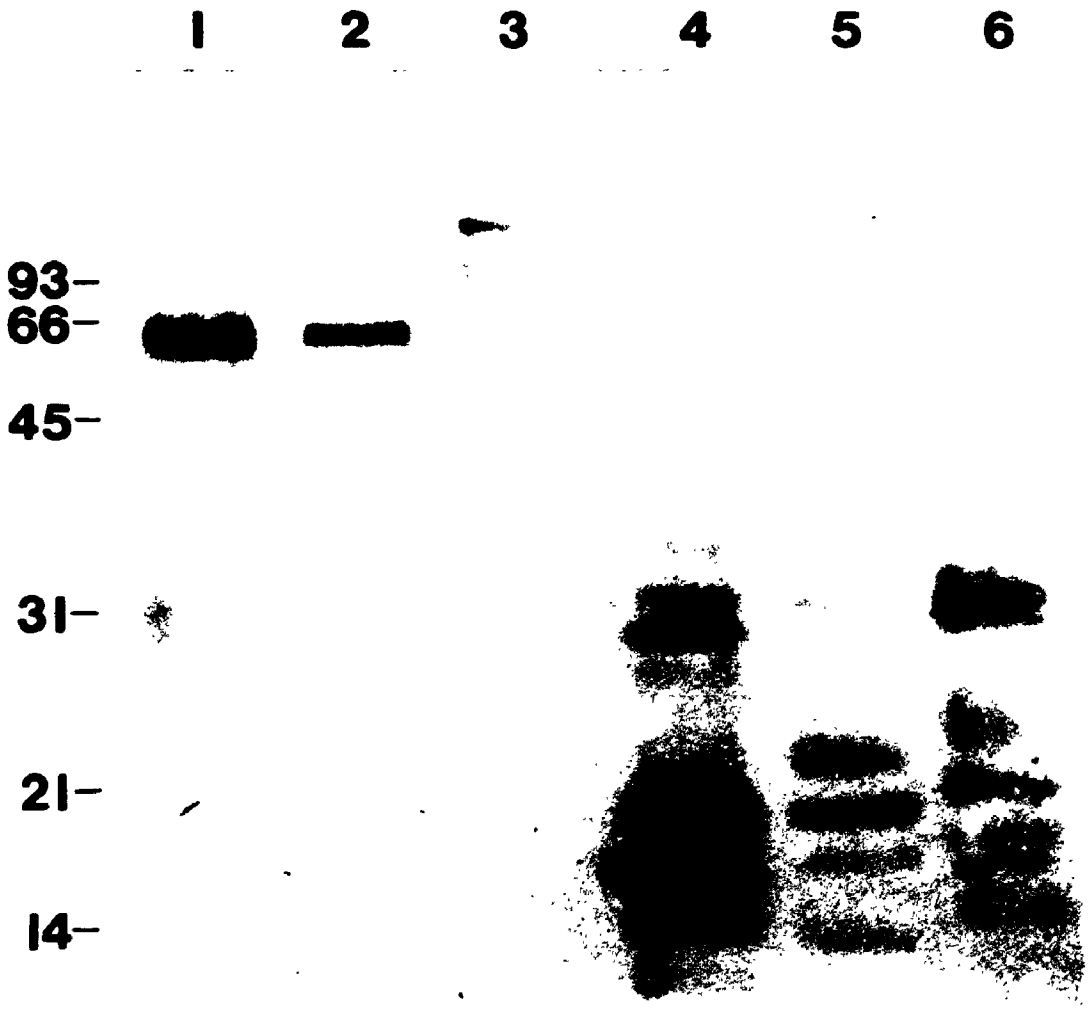
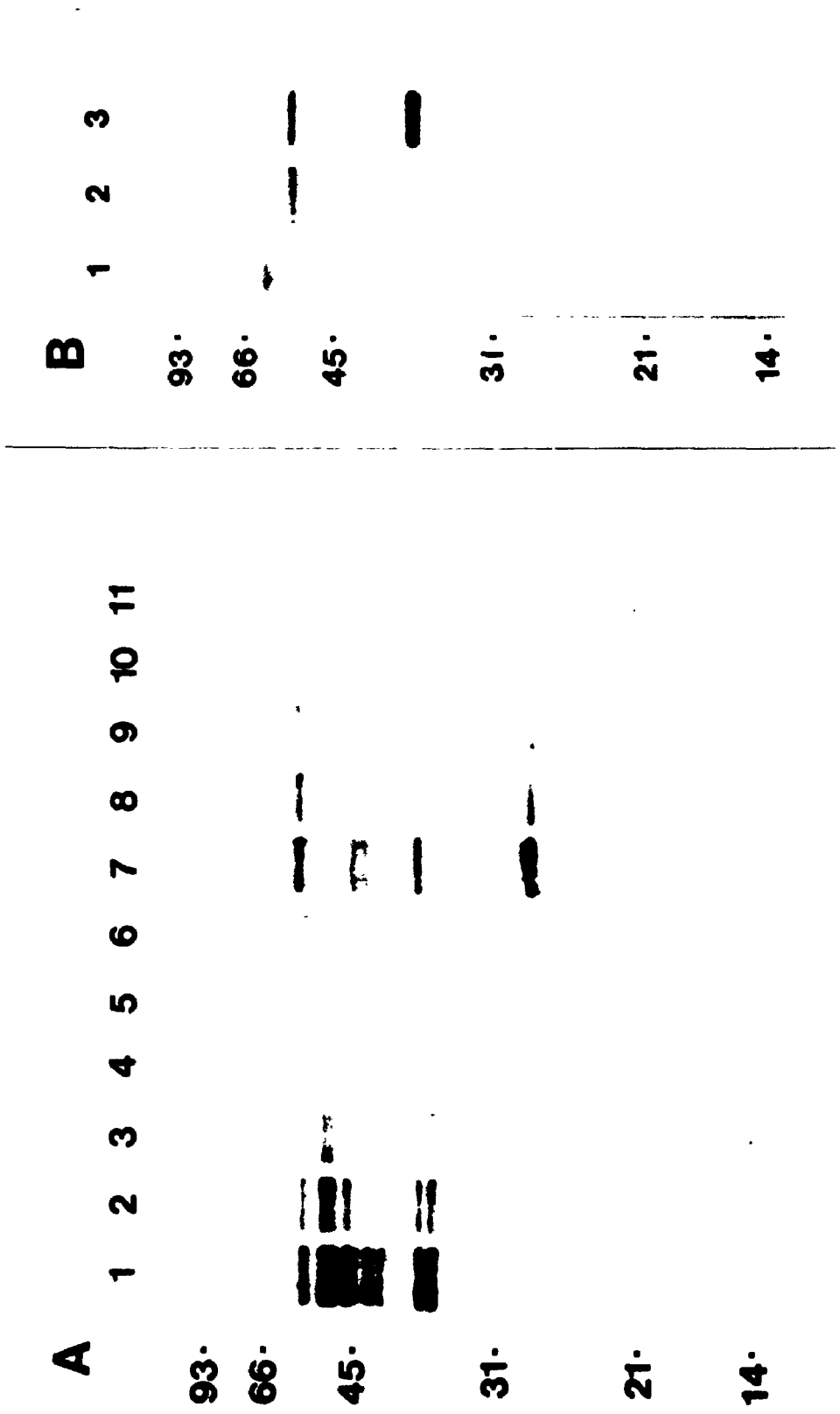

1
2
3
4
5
6
93–
66–
45–
31–
21–
14–

Figure 4.5: Expression of WaTat 1.13 rVSG from pT7-7.13. A) Comparison of expression levels observed from p14 and pT7-7.13. Lysates from clones p14 (lanes 1-5) and pT7-7.13 (lanes 7-11) were resolved by 12% SDS PAGE, semi-dry transferred to immobilon-P membranes and probed with MAb P14B1. Lysates were prepared from induced cultures grown to the same density. Cells from 100 μl of equally dense induced cultures were pelleted then lysed in sample buffer. Equivalents of 10, 5, 2, 1 and 0.5 μl of lysates were loaded for each series. 10 ng WaTat 1.13 sVSG was electrophoresed in lane 6. B) Instability of WaTat 1.1. expression from pT7-7.13. 12% SDS PAGE western blot analysis of purified WaTat sVSG (lane 1), pT7-7.13 lysate prepared immediately after transformation (lane 2) and pT7-7.13 lysate prepared 3 days post-transformation after storage as a colony on a LB + ampicillin agar plate at 4°C. Lysates were prepared in an identical manner and the blot was probed with MAb P14B1. Positions of molecular weight markers are indicated (in kDa) to the left of each blot.


B
1
2
3
93
66
45
31
21
14
A
1
2
3
4
5
6
7
8
9
10
11
93
66
45
31
21
14

were obtained with the plasmid pGEMEX-1. Although expression from pGEMEX-1 is also based on a T7 polymerase system, it is induced by IPTG and, consequently, expression appears to be less "leaky" under non-induced conditions. cDNAs for WaTats 1.12 and 1.14 were inserted into pGEMEX-1 (Figure 4.3C & D) and clones expressing respective VSGs were isolated as described above. These sub-clones were designated pGEMEX.12 and pGEMEX.14. WaTat 1.14 rVSG expressed from pGEMEX.14 was detectable on western blots as a band of M_r 110 kDa (Figure 4.4, lane 3). Its high M_r is due to the presence of 260 amino acids of the T7 gene 10 product which is fused to the VSG's N-terminus. Cleveland mapping of the expressed product (Figure 4.4, lane 6) revealed a pattern of immunoreactive fragments similar to those seen for purified WaTat 1.14 sVSG and rVSG expressed from p14 (Figure 4.4, lanes 4 & 5 respectively). It should be noted that band intensities of expressed products shown in Figure 4.4 are not indicative of expression levels since loadings of bacterial lysate from p14 and pGEMEX.14 clones are not equivalent. Rather, in comparative experiments, the expression level from pGEMEX.14 was approximately 10 fold greater than from p14.

4.3.2.2 Exonuclease III Deletions

The start points and directions of exo III deletions created in the various expression constructs are illustrated in Figure 4.3. Exo III will progressively degrade double stranded DNA starting from a 5' overhang or blunt end. In order to ensure unidirectional deletions into the insert, the DNA must be double cut with restriction enzymes such that a 5' overhang or blunt end exists on the insert side

and a 3' overhang remains on the vector side. Restriction sites used for double cutting are indicated at the exo III start points in Figure 4.3. 3' to 5' deletions created in p14 were used to map C-terminal-most boundaries of pan-specific epitopes. Since poor expression levels from p14 precluded demarcation of C-terminal-most boundaries of P14A1/P14B1 and P13A2 epitopes, 3' to 5' deletions were created in pGEMEX.14 and screened. In order to define N-terminal-most boundaries of pan specific epitopes, 5' to 3' deletions were created in pGEMEX.14. Screening these 5' to 3' deletion clones was particularly arduous because reading frame was disrupted ie. only 1 out of every 3 deletion clones was expressing VSG sequence. Extreme and mysterious difficulties were encountered during attempts to create 3' to 5' deletions in pGEMEX.12. Consequently, sub-pan specific determinants were localized from 5' to 3' deletions in this construct.

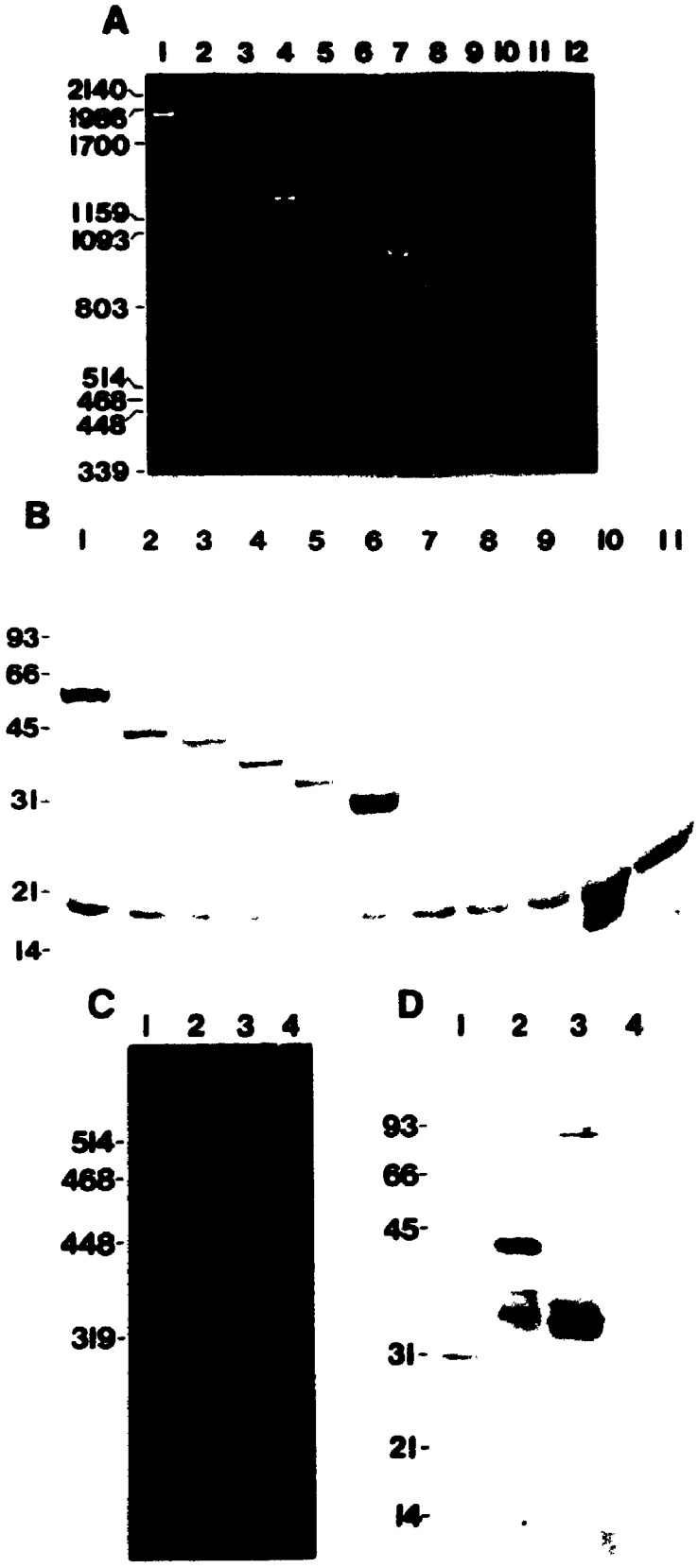
4.3.2.3 Examples of Epitopes Localized by Deletion Analysis

Since it would be impractical to present the results of deletion clone screens for all MAbs (approximately 60 gels total), following are results for three of the most interesting MAbs.

4.3.2.3.1 Localization of an N-Terminal Epitope; P14B1

DNA restriction fragments indicating size of insert remaining in low resolution deletion clones from the p14 3' to 5' deletion series, are shown in Figure 4.6A. These clones span the entire WaTat 1.14 cDNA and are approximately equally spaced. When P14B1 is used to probe lysates from induced cultures on western blots (Figure 4.6B), it recognizes all of the deletion clones down to the

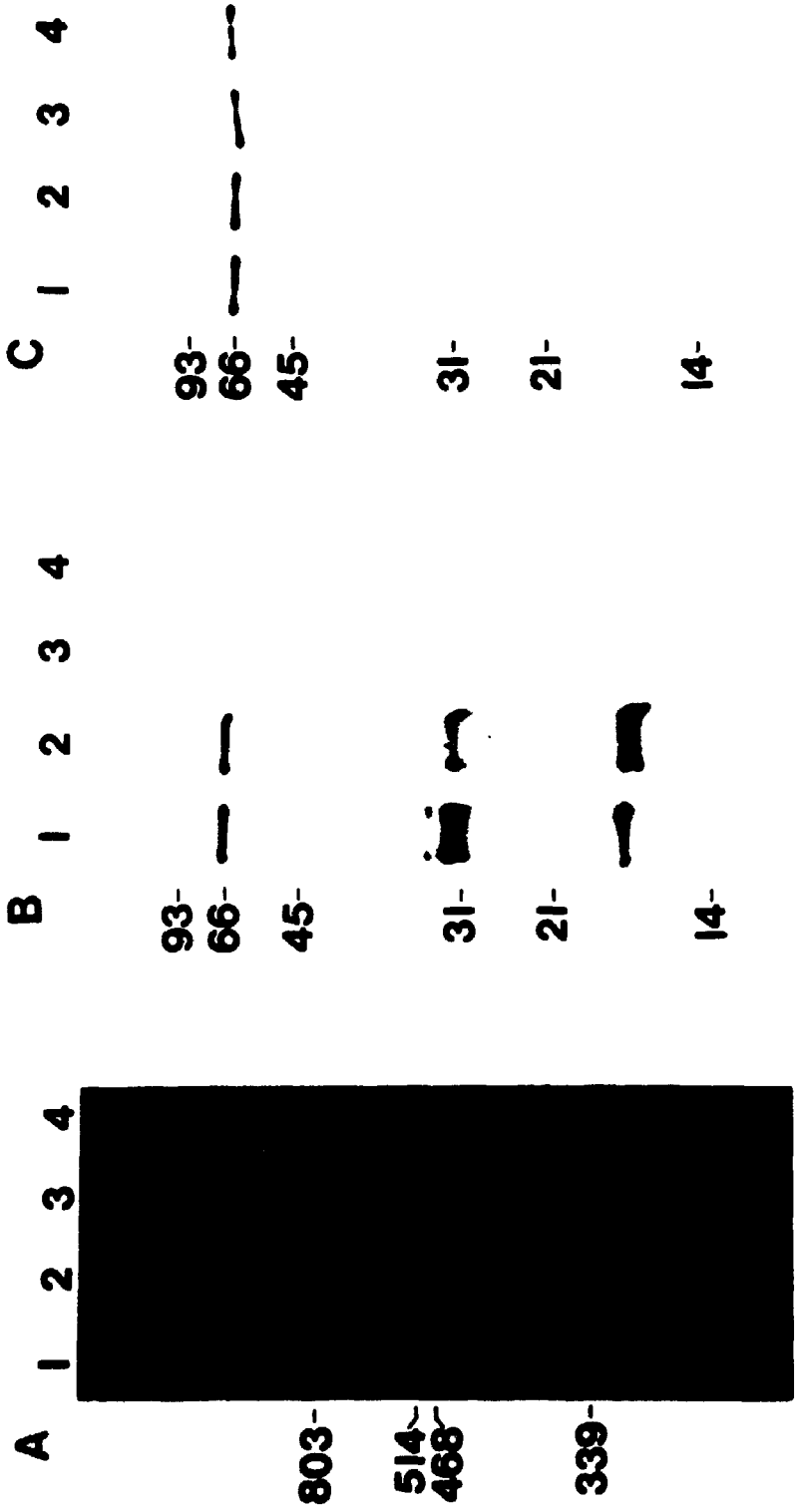
Figure 4.6: Localization of the C-terminal-most boundary of an N-terminal epitope (P14B1). A) Hind III/Eco RI restriction fragments of 3'-5' p14 low resolution deletion clones separated by electrophoresis in 1.5% agarose and stained with ethidium bromide. B) Western blot analysis of 3'-5' p14 low resolution deletion clone lysates separated on 10-15% Fling gels and probed with P14B1. Lane assignments for A and B, lanes 1-11 are as follows: p14, 4X, 7B, 10W', 10V', 13D, 16H, 19A, 19C, 22X and 4C. Lane 12 in A, pBR322 stops fragment. C) Kpn I/Bsp EI restriction fragments of 3'-5' pGEMEX.14 high resolution deletion clones separated on a 4% PAGE gel stained with ethidium bromide. D) Western blot analysis of 3'-5' pGEMEX.14 high resolution deletion clones separated 12% SDS PAGE and probed with P14B1. Lane assignments for C and D are as follows: lane 1, N191; lane 2, N93; lane 3, M37 and lane 4, M89. Positions of standard markers are indicated to the left of each gel: A & C, Pst cut lambda (in base pairs); B & D, BioRad low molecular weight standards (in kDa). See also text for description of restriction fragment sizes from the DNA gel in D.


A
1 2 3 4 5 6 7 8 9 10 11 12
2140
1986
1700
1159
1093
803
514
468
448
339
B
1 2 3 4 5 6 7 8 9 10 11
93
66
45
31
21
14
C
1 2 3 4
514
468
448
319
D
1 2 3 4
93
66
45
31
21
14

smallest, 4C (lane 11), which encodes approximately 30 amino acids at the extreme N-terminus. Due to difficulties in obtaining high level expression of p14 deletion clones in this area, 3' to 5' high resolution deletion clones were isolated from a deletion series created in pGEMEX.14. PAGE resolved restriction fragments were faint on the original gel and consequently are difficult to see in Figure 4.6C. Their sizes are, therefore, presented here as follows: N93 (lane 1), 371 bp; N19 (lane 2), 333 bp; M37 (lane 3), 291 bp and M89 (lane 4), 287 bp. These restriction fragments contain approximately 230 bp of vector sequence in addition to VSG sequence. When probed with P14B1 on western blots (Figure 4.6D) all are positive except for the smallest, M89 (lane 4). Although M89 was positive on patch blots probed with P14B1 it may have lost expression prior to the western blot screen.

High resolution deletion clones spanning the N-terminal region were isolated from the pGEMEX.14 5' to 3' deletion series (Figure 4.7). When these clones are probed with P14B1 on western blots (Figure 4.7B), reactivity is lost between clones A16 (lane 2) and A141 (lane 3). As a positive control, these clones were also probed with MAb P12A1, which binds to a downstream epitope (Figure 4.7C). This clearly demonstrated that all clones, including A141, were expressing deletion products. Determined by DNA sequencing, A16 was deleted to the mature N-terminus and the junction for A141 was found to be 4 amino acids further into the VSG sequence. Because A16 is only at the immediate N-terminus, the size of the region harbouring the P14B1 antigenic determinant was not narrowed any further than from the 3' to 5' deletion analysis ie. it remains at 25 amino acids. As with

Figure 4.7: Localization of the N-terminal-most boundary of an N-terminal antigenic determinant (P14B1). A) Kpn I/Sty I restriction fragments from 5'-3' pGEMEX.14 high resolution deletion clones separated by 4.0% PAGE and stained with ethidium bromide. B) & C) 12% SDS PAGE western blot analysis of 5'-3' pGEMEX.14 high resolution deletion clones probed with P14B1 (B) and P12A1 (+ve control, C). lanes 1-4: B33, A16, A141 and A162 respectively, for all three gels. Positions of Pst cut lamoda markers (in base pairs) and BioRad low molecular weight markers (in kDa) are indicated to the left of the DNA gel and the western blots respectively.


A
1 2 3 4
803-
514
468
339-
B
1 2 3 4
93-
66-
45-
31-
21-
14-
C
1 2 3 4
93-
66-
45-
31-
21-
14-

many of the deletion clones examined on western blots, breakdown products are detectable. This appears to be a common problem with expression of foreign sequences and especially deletion products (eg. Dowbenko *et al.*, 1988; Gill *et al.*, 1988; Miller *et al.*, 1989)

4.3.2.3.2 Localization of a Central Epitope; P13C2

When P13C2 is used to probe 3' to 5' low resolution clones from p14 (Figure 4.8B), its corresponding epitope is defined as being between clones 16H and 19A. When high resolution deletion clones spanning this region are isolated and screened (Figure 4.8E) with P13C2, 16.3 (lane 3) and 16.23 (lane 4) appear to define the P13A2 epitope's C-terminal-most boundary. Expression of all low and high resolution deletion clones was confirmed with other MAbs (Figure 4.8C & F respectively). From DNA sequencing it was determined that the 16.3 junction mapped to amino acid position 204 of the mature polypeptide and 16.23 was deleted to two amino acids further towards the N-terminus.

To define the N-terminal most boundary, P13C2 was used to probe high resolution clones from the 5' to 3' deletion series of pGEMEX.14 (Figure 4.9B). The definitive clones, C352 and C378, were also determined to be two amino acids apart at positions 198 and 200. Combining the information generated from deletion analysis in both directions, the antigenic determinant recognized by P13C2 has been defined to 6 amino acids.

4.3.2.3.3 Localization of a Sub-Pan Specific Epitope; P12B1

Since sub-pan specific MAbs do not recognize the WaTat 1.14 variant (see

Figure 4.8: Localization of the C-terminal-most boundary of a central epitope (P13C2). A) Ethidium bromide stained 1.5% agarose gel of resolved Hind III/Eco RI restriction fragments from 3'-5' p14 low resolution deletion clones. B) & C) 10-15% Fling gradient SDS PAGE western blots of lysates from 3'-5' p14 low resolution deletion clones probed with P13C2 (B) and P14B1 (+ve control, C). For A, B and C lanes 1-11: p14, 4X, 7B, 10W', 10V', 13D, 16H, 19A, 19C, 22X and 4C respectively. A, lane 12 contains pBR322 stops fragment. D) 4.0% PAGE gel stained with ethidium bromide of Hind III/Eco RI restriction fragments from 5'-3' p14 high resolution deletion clones. E) & F) 12% SDS PAGE western blots of 5'-3' p14 high resolution deletion clones probed with P13C2 (E) and P14B1 (+ve control, F). lanes 1-5: 16.13, 16.3, 16.23, 16.7 and 16.20 respectively. Positions of Pst cut lambda markers (in base pairs) and BioRad low molecular weight standards (in kDa) are indicated to the left of the DNA gels and western blots respectively.

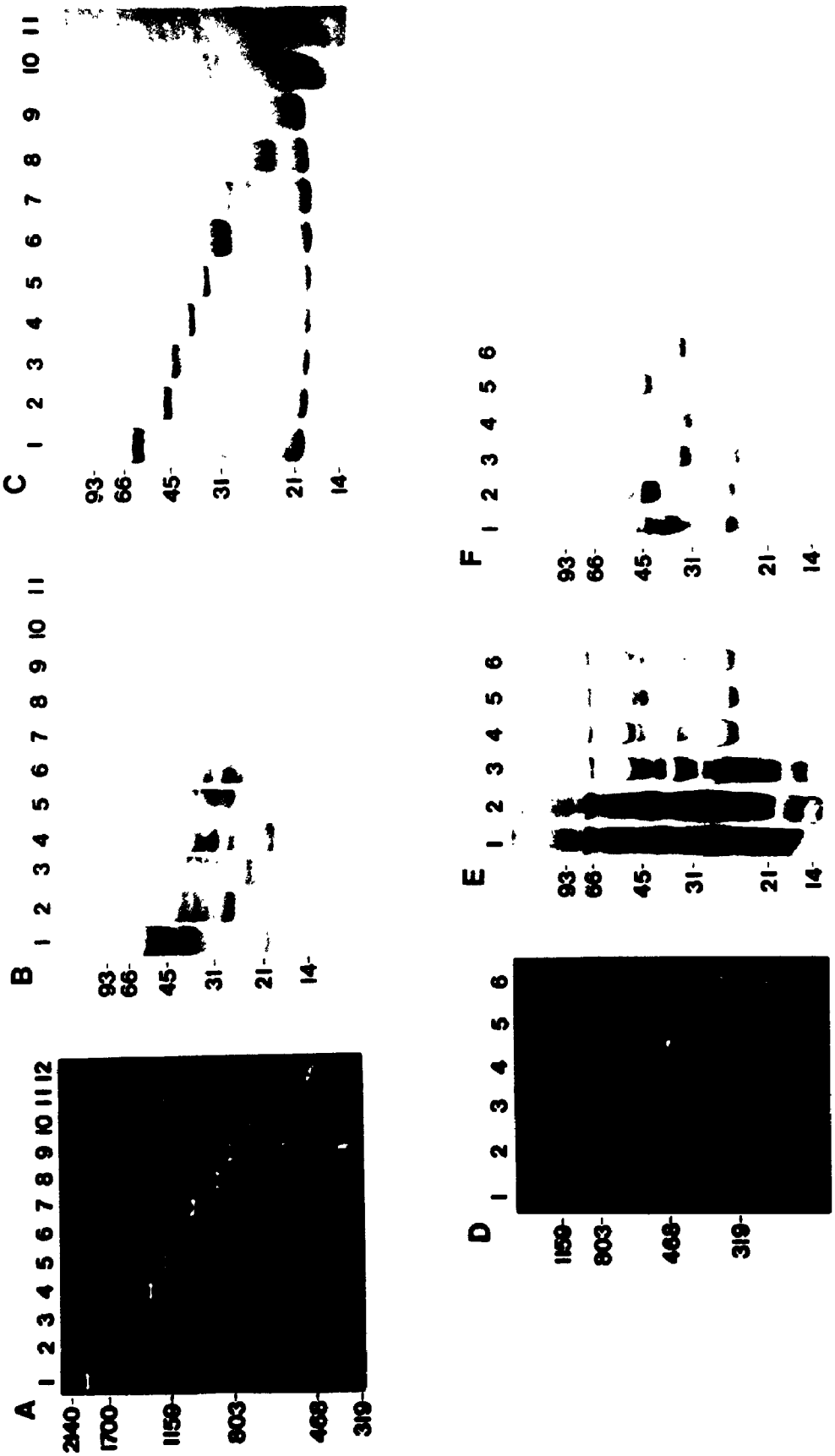

A
1 2 3 4 5 6 7 8 9 10 11 12
2340
1700
1159
803
468
319
B
1 2 3 4 5 6 7 8 9 10 11
93
66
45
31
21
14
C
1 2 3 4 5 6 7 8 9 10 11
93
66
45
31
21
14
D
1 2 3 4 5 6
1159
803
468
319
E
1 2 3 4 5 6
93
66
45
31
21
14
F
1 2 3 4 5 6
93
66
45
31
21
14

Figure 4.9: Localization of the N-terminal-most boundary of a central epitope (P13C2). A) Ethidium bromide stained Kpn I/Pst I restriction fragments from 5'-3' pGEMEX.14 high resolution deletion clones resolved on a 4.0% PAGE gel. B) & C) 12% SDS PAGE western blots of 5'-3' pGEMEX.14 high resolution deletion clones probed with P13C2 (B) and P12A1 (+ve control, C). Lane assignments for all gels are as follows: C377 (lane 1), C237 (lane 2), C274 (lane 3), C352 (lane 4), C378 (lane 5) and C325 (lane 6). Positions of Pst I restricted lambda markers (in base pairs) and BioRad low molecular weight standards (in kDa) are indicated to the left of the DNA gel and western blots respectively.

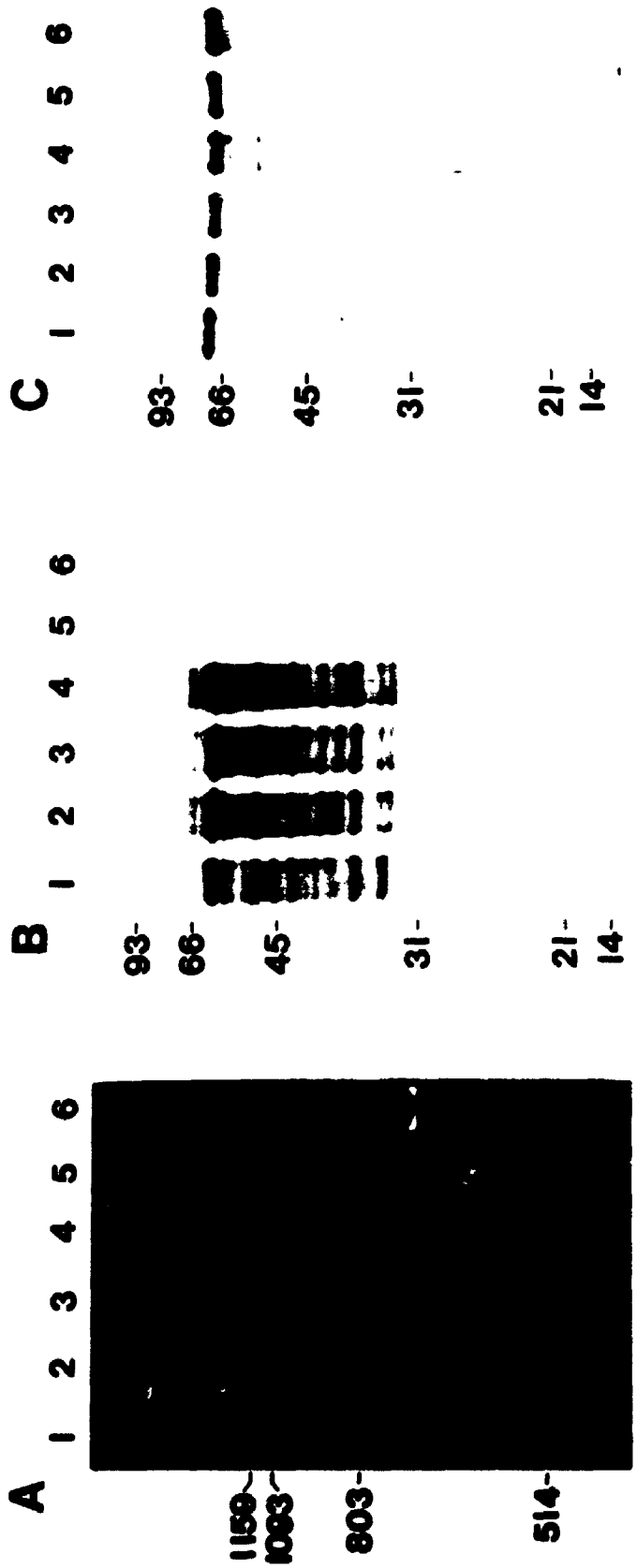

A
1 2 3 4 5 6
1159
1083
803
514
B
1 2 3 4 5 6
93
66
45
31
21
14
C
1 2 3 4 5 6
93
66
45
31
21
14

Figure 3.2), analysis of their corresponding epitopes was performed using pGEMEX.12. However, as described in section 4.3.2.2, difficulties encountered during attempts to create 3' to 5' deletions in pGEMEX.12 necessitated analysis in the 5' to 3' direction only.

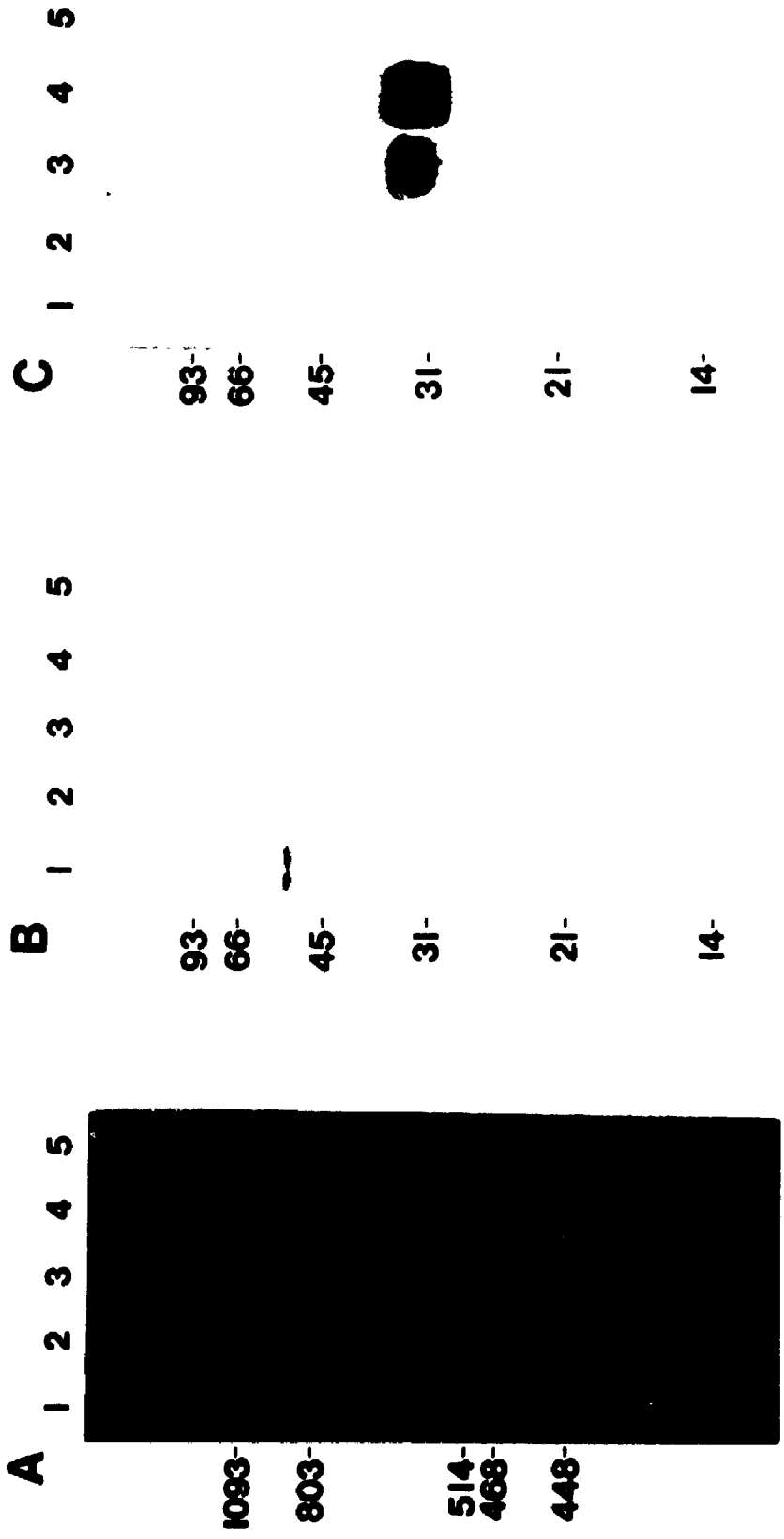
DNA restriction fragments from the smallest P12B1 immunoreactive 5' to 3' deletion clones examined are shown in Figure 4.10A. Bands are again difficult to see, therefore, their sizes are presented here as follows: P456 (lane 1), 810 bp; P369 (lane 2), 465 bp; P408 (lane 3), 460 bp; P321 (lane 4), 460 bp and P425 (lane 5), 450 bp. When probed with P12B1 on western blots (Figure 4.10C), the two smallest positive clones, P408 and P321 (lanes 3 and 4), appear to be identical in size. Sequencing of these clones defined the P12B1 epitope to within the last 43 amino acids of the mature C-terminus.

P456 was included in the analysis because it exhibited unique results ie. it was P12A1 positive but P12B1 negative on patch blots. This was unusual because all of the other clones examined by patch screening were P12A1 negative but P12B1 positive, suggesting that the P12B1 epitope was downstream from P12A1. Analysis on western blots (Figure 4.10B & C, lane 1 for both) confirms the anomalous result. More recently, it was determined that P456 is from the 5'-3' deletion series of pGEMEX.14. Since P12A1 (control antibody) can recognize WaTat 1.14 VSG but P12B1 cannot, the basis of the unexpected result is apparent.

4.3.2.4 Location and Structure of Epitopes Recognized by Monoclonal Antibodies

Positions of deletion clones defining borders of each epitope are illustrated

Figure 4.10: Localization of the N-terminal-most boundary of a sub-pan specific epitope (P12B1). A) 4.0% PAGE gel stained with ethidium bromide of Kpn I/Bsp EI restriction fragments from 5'-3' pGEMEX.12 high resolution deletion clones. B) & C) Lysates of 5'-3' pGEMEX.12 high resolution deletion clones analysed on 12% SDS PAGE western blots probed with P12A1 (B) and P12B1 (C). Lane assignments for all gels are as follows: Q3 (lane 1), O202 (lane 2), Q19 (lane 3), Q498 (lane 4) and O318 (lane 5). Positions of Pst I restricted markers (in base pairs) and BioRad low molecular weight standards (in kDa) are indicated to the left of the DNA gel and western blots respectively. See text for the description of restriction fragment sizes from the DNA gel.


A
1 2 3 4 5
1093
803
514
468
448
B
1 2 3 4 5
93
66
45
31
21
14
C
1 2 3 4 5
93
66
45
31
21
14

in Figure 4.11. In each case, positions of clones having the smallest length of insert remaining, yet still recognized by the corresponding MAb (last +ves) are given. Similarly presented are the positions of the largest negative clones (first -ves). Binding specificity of each MAb is lost somewhere between the last +ve and first -ve in each direction. This suggests that one or more amino acids between the two points is/are critical to the integrity of the antigenic determinant. For example, with the N-terminal most boundary of P13C2, the codon for Gly^{199} is still intact for the last +ve clone (C352), however, the codons for Gly^{199} and Phe^{200} have been deleted from C358 which is -ve, therefore Gly^{199} and/or Phe^{200} are critical residues. It should be noted that delineation of epitope boundaries by this analysis does not preclude the possible existence of flanking residues which significantly interact with the antibody paratope. However, under the assay conditions in which antibody/antigen interactions were examined (western blot), such residues are not critical for binding.

Collectively, the antigenic determinants for a total of 11 MAbs have been localized to varying degrees in this study. These MAbs bind to at least 7 discrete sites distributed throughout the linear VSG sequence (Figure 4.12). Epitopes exist at both the extreme N- and C-termini (P14A1/P14B1 and P12B1/P12C1 respectively) and are loosely clustered throughout the central region (all remaining MAbs). In addition to location, the structure of each determinant is also presented in Figure 4.12.

Figure 4.11: Positions of deletion clones that define the boundaries of antigenic determinants. A) P14A1/P14B1. B) P13A2. C) P13C2. D) V13B1/V13C5. E) P12A1 F) P12B1/P12C1. Indicated for each epitope is the DNA sequence, the corresponding amino acid sequence and the positions of deletion clones which define the boundaries of the site. Arrows point to the last base of VSG sequence remaining in the corresponding deletion clone. All sequences are from variant 1.14 except for the P12B1/P12C1 epitope which is from WaTat 1.12. // is used to signify a break in the sequence if the epitope was too long to be displayed in the figure. Positions of amino acids, relative to the mature N-terminus, are indicated above the terminal residues for each antigenic site.

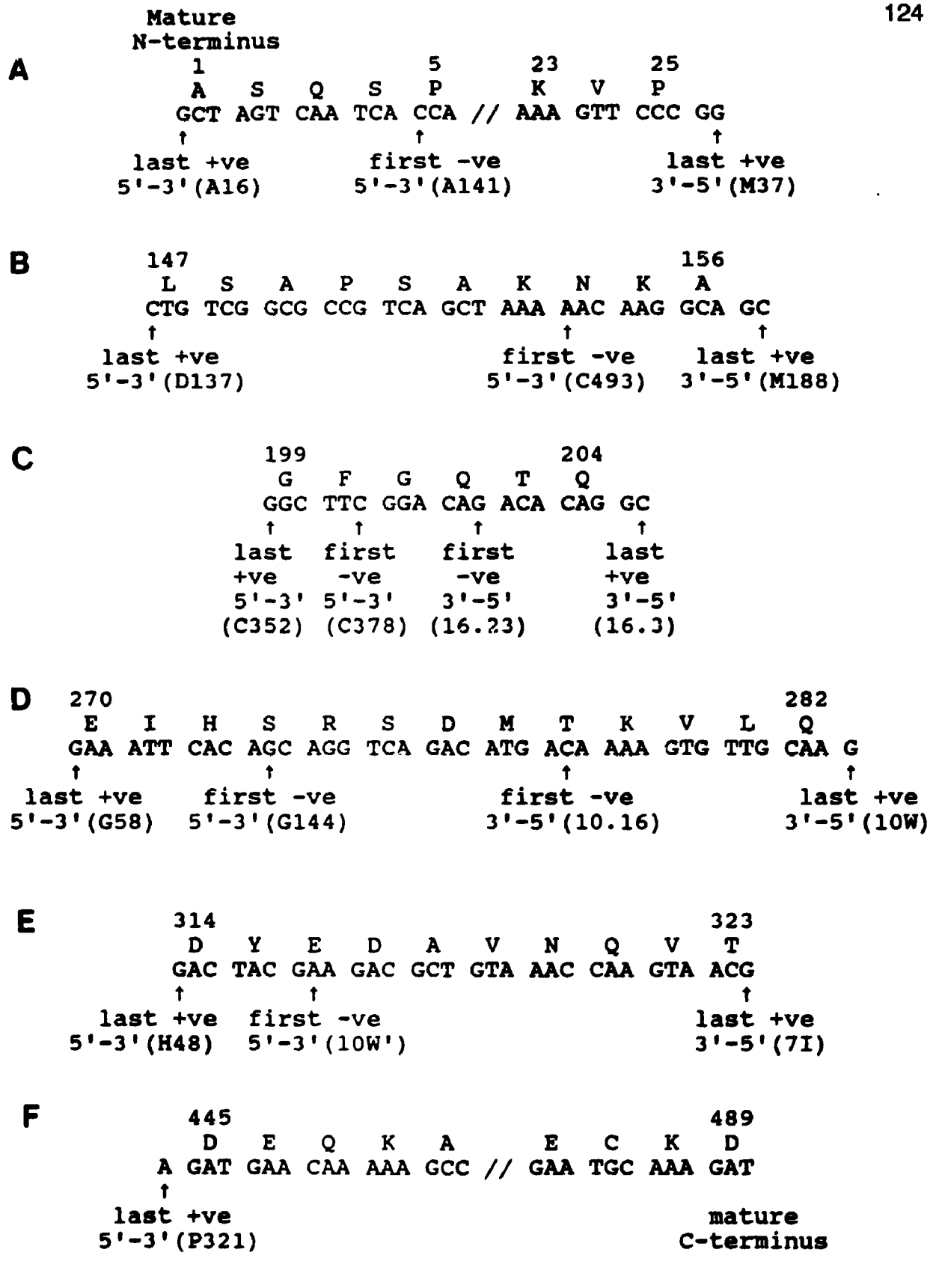
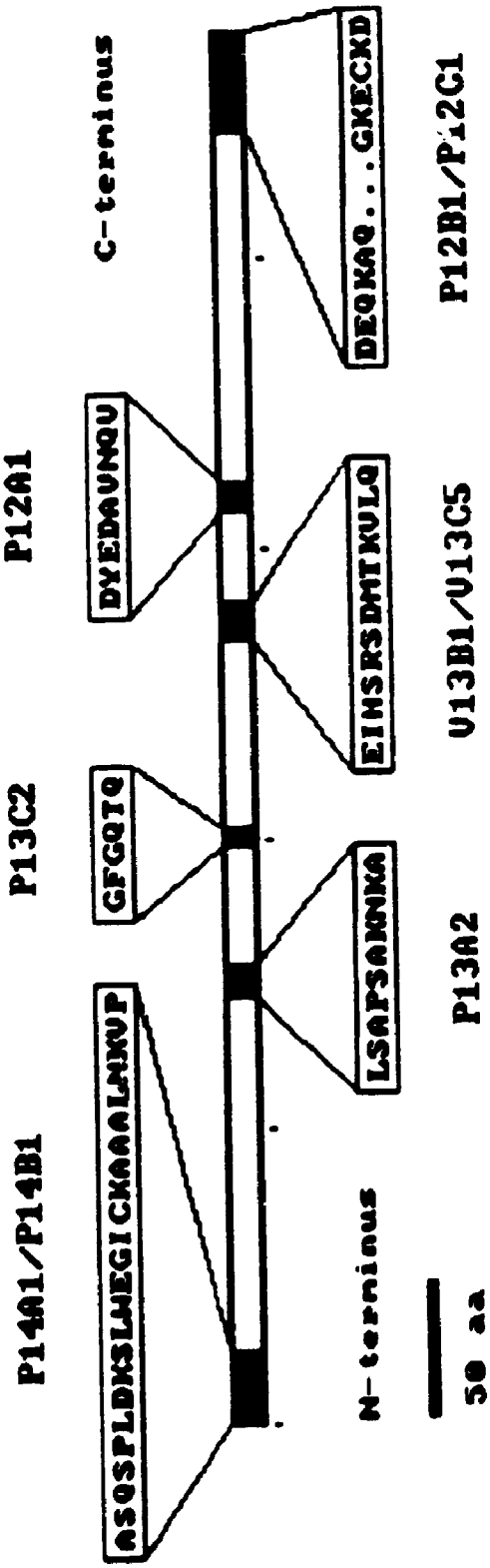

A
Mature
N-terminus
1 5 23 25
A S Q S P K V P
GCT AGT CAA TCA CCA // AAA GTT CCC GG
last +ve
5'-3'(A16)
first -ve
5'-3'(A141)
last +ve
3'-5'(M37)
B
147 156
L S A P S A K N K A
CTG TCG GCG CCG TCA GCT AAA AAC AAG GCA GC
last +ve
5'-3'(D137)
first -ve
5'-3'(C493)
last +ve
3'-5'(M188)
C
199 204
G F G Q T Q
GGC TTC GGA CAG ACA CAG GC
last +ve
5'-3'
(C352)
first -ve
5'-3'
(C378)
first -ve
3'-5'
(16.23)
last +ve
3'-5'
(16.3)
D
270 282
E I H S R S D M T K V L Q
GAA ATT CAC AGC AGG TCA GAC ATG ACA AAA GTG TTG CAA G
last +ve
5'-3'(G58)
first -ve
5'-3'(G144)
first -ve
3'-5'(10.16)
last +ve
3'-5'(10W)
E
314 323
D Y E D A V N Q V T
GAC TAC GAA GAC GCT GTA AAC CAA GTA ACG
last +ve
5'-3'(H48)
first -ve
5'-3'(10W')
last +ve
3'-5'(7I)
F
445 489
D E Q K A E C K D
A GAT GAA CAA AAA GCC // GAA TGC AAA GAT
last +ve
5'-3'(P321)
mature
C-terminus

Figure 4.12: Locations of antigenic determinants recognized by monoclonal antibodies. Positions of epitopes bound by MAbs are indicated by black areas on the VSG which is schematically represented as an open black bar with the N-terminus at the left and the C-terminus to the right. Positions of amino acids are indicated at 100 amino acid intervals relative to the mature N-terminus. Amino acid sequences of antigenic sites are displayed in the expanded boxes and in each case are from the WaTat 1.14 VSG except for the P12B1/P12C1 epitope which is from variant 1.12.

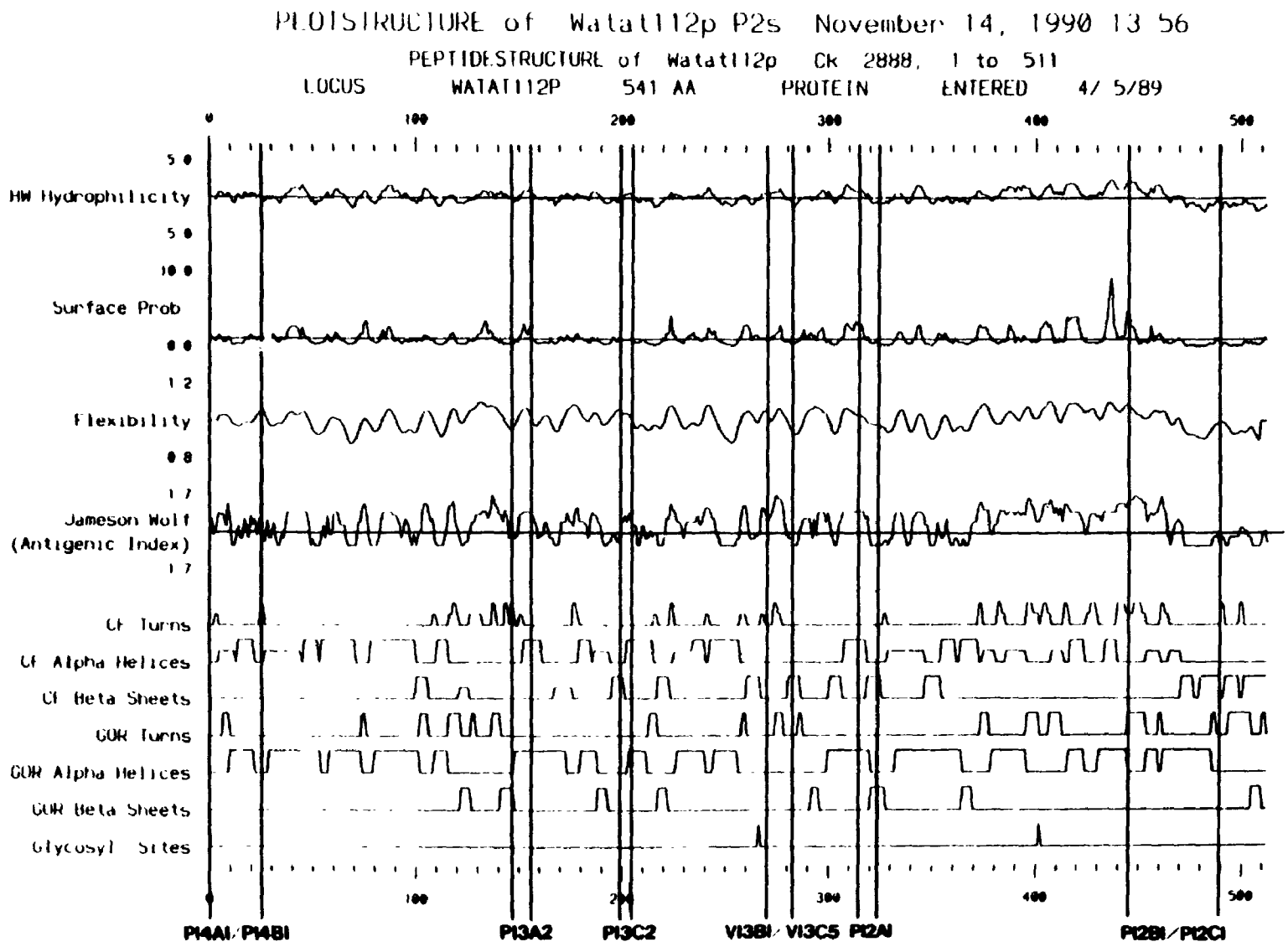

P14A1/P14B1
P13C2
P12A1
C-terminus
ASQSPLDKSLWEGICKAAALAKWP
GFGQTQ
DYEDAVNQV
DEQKAQ...GKECKD
LSAPSAKNKA
EIHSRSDMTKVLQ
N-terminus
P13A2
U13B1/U13C5
P12B1/P12C1
50 aa

4.3.2.5 Epitope Positions; Computer Predictions Versus Experimental Results

Presented in Figure 4.13 are the results from analysis of the WaTat 1.12 protein sequence by several computer prediction programs. Illustrated are profiles representing hydrophilicity, surface probability, flexibility, antigenicity, secondary structure characteristics and sites for potential glycosylation along the protein sequence. However, I will only refer to the results of the antigenic index (AI) prediction program (Devereux, 1984) since it is most pertinent to the discussion of epitopes. As can been seen in Figure 4.13, a moderate correlation exists between antigenic sites localized experimentally and AI measurements. In some cases, such as for P13C2 and V13B1/V13C5, AI peaks are present in the middle of antigenic determinants - although the peak for P13C2 is small. However, in all other instances, relatively low AI measurements can be found at some point in the experimentally localized determinants. It could be argued that this program was designed to predict solvent exposed epitopes and therefore only these sites should be regarded. Taking this into consideration (see also section 4.3.2.7), sites for P13C2 and V13B1/V13C5 contain AI peaks but a sharp decline in AI is seen within the P12A1 site. Although data is limited in this comparative analysis, I would estimate the correlation between experimental and predicted results to be approximately 60-70%.

Of interest to the V13B1/V13C5 antigenic determinant, is the predicted glycosylation site present only 4 amino acids from its N-terminal-most boundary. As concluded from the deglycosylation studies (section 2.2.6), this site is indeed

Figure 4.13: Structural characteristics of the WaTat 1.12 sVSG predicted by computer analysis. Illustrated is the output from a program called Plotstructure which plots the data generated from analysis of the VSG sequence by the Peptidestructure program. Both programs are part of the Genetics Computer Group (GCG) sequence analysis package. The sequence analysed was from the mature N-terminus to the stop codon which includes the C-terminal hydrophobic tail, not present on the mature polypeptide. The boundaries of antigenic determinants are indicated by vertical lines running across all of the profiles. HW, Hopp and Woods; CF, Chou and Fasman; GOR, Garnier, Osguthorpe, and Robson are all authors of the respective algorithms.


PLOTSTRUCTURE of Watat112p P2s November 14, 1990 13 56
PEPTIDESTRUCTURE of Watat112p Ck 2888, 1 to 511
LOCUS WATAT112P 541 AA PROTEIN ENTERED 4/ 5/89
0
100
200
300
400
500
5 0
HW Hydrophilicity
5 0
10 0
Surface Prob
0 0
1 2
Flexibility
0 8
1 7
Jameson Wolf
(Antigenic Index)
1 7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl Sites
PI4AI/PI4BI
PI3A2
PI3C2
VI3BI/VI3C5
PI2AI
PI2BI/PI2CI

glycosylated. This modification, however, does not affect antibody binding, suggesting that the oligosaccharide is somehow oriented away from the antigenic site, thereby avoiding any steric hindrance to the antibody.

4.3.2.6 The Effects of Amino Acid Substitutions on the Immunospecificity of Antigenic Determinants

By comparing amino acid sequences within antigenic sites on isoVAT VSGs, a number of both allowable and non-allowable substitutions, depending on their occurrence in pan and sub-pan specific epitopes respectively, can be observed (Figure 4.14).

Beginning with pan-specific epitopes, three positions in the first 15 amino acids of the extreme N-terminus, which probably constitutes the P14A1/P14B1 epitope, contain allowable substitutions. Clearly the most radical of the substitutions is the Ala/Pro replacement occurring at position 5. Proline, being an imino acid, has a unique structure which tends to put a kink in the alpha carbon backbone at its position. However, this marked structural alteration appears to have no effect on antibody binding. Of somewhat less significance is the next substitution, which occurs at position 9, Gly/Ser, since both are small amino acids. To the contrary, although the Gln to Glu replacement at position 12 represents very little change in size, these amino acids differ significantly in charge.

Although the antigenic determinant recognized by P13C2 is only 6 amino acids in length, two substitutions, at positions 4 and 6 of the site, have occurred. Both are Gln/Glu replacements which represent charge differences as noted

Figure 4.14: Substitutions occuring within antigenic determinants on WaTat isoVAT VSGs. For each antigenic site, the sequence of WaTat 1.1 is shown in the standard single letter code. For WaTat 1.12, WaTat 1.13, and WaTat 1.14 sequences, at each position, identity with WaTat 1.1 is indicated by a "-" while substitutions are indicated by the single letter code for replacement amino acids. Gaps introduced to optimize the alignment are indicated by periods (.).

P14A1/P14B1

```
WaTat 1.1  ASQSALDKGLWQGICKATAELNKVP
WaTat 1.12 -----------E----------A--
WaTat 1.13 ----P---S----------------
WaTat 1.14 ----P---S--E-----A-Q-----
```

P13A2

```
WaTat 1.1  LSAPSAKNKA
WaTat 1.12 ----------
WaTat 1.13 ----------
WaTat 1.14 ----------
```

P13C2

```
WaTat 1.1  GFGQTE
WaTat 1.12 ---E--
WaTat 1.13 ---E-Q
WaTat 1.14 -----Q
```

V13B1/V13C5

```
WaTat 1.1  EIHSRSDMTKVLQ
WaTat 1.12 -------------
WaTat 1.13 -----P-------
WaTat 1.14 -------------
```

P12A1

```
WaTat 1.1  DYEDAVNQQVTI
WaTat 1.12 ------------
WaTat 1.13 ------------
WaTat 1.14 ------------
```

P12B1/P12C1

```
WaTat 1.1  DEQKAQGAGTGDGAAGEQKKEDKCTGKKKDD.CKD.GCKWEGKECKD
WaTat 1.12 ------------------------------.---.-----------
WaTat 1.13 ----------------------KD-----.---.SPD---ET---
WaTat 1.14 EGKTNTA-.....---TNSEGK--SD--SEVV---.----D-T----
```

above. Interestingly, either or both Thr and/or Gln at positions 5 and 6 respectively, are critical to maintaining the integrity of the epitope, since they exist between the last +ve and first -ve deletion clones which define the C-terminal most boundary. The Gln/Glu substitution at position 6 may suggest that it is a non-contact residue, thereby highlighting the importance of threonine at position 5. Furthermore, in the first -ve clone (16.23), the amino acid present, at what would be position 5, is aspartic acid, suggesting that this substitution is non-allowable.

The only substitution occurring in the V13B1/V13C5 epitope is another replacement involving proline, this time substituted for alanine at position 6. Again this represents a radical substitution that doesn't affect specificity.

Only one epitope of the sub-pan specific variety was localized: P12B1/-P12C1, within the last 45 amino acids of the C-terminus. These MAbs do not recognize variants 1.13 and 1.14, suggesting that non-allowable substitutions have occurred somewhere in this region. The only positions at which substitutions occur in both 1.13 and 1.14 are residues 25, 26 and 41. This suggests these positions may be critical for specificity, however, without any knowledge of higher ordered structure characteristics of the polypeptide in this region, it is impossible to discount the possibility of effects due to independent substitutions at other positions.

4.3.2.7 Solvent Accessibility of Epitopes Determined by Solution Phase Antibody Inhibition Assays Using Homologous VSG

Although MAbs used in this study were selected by their ability to bind to

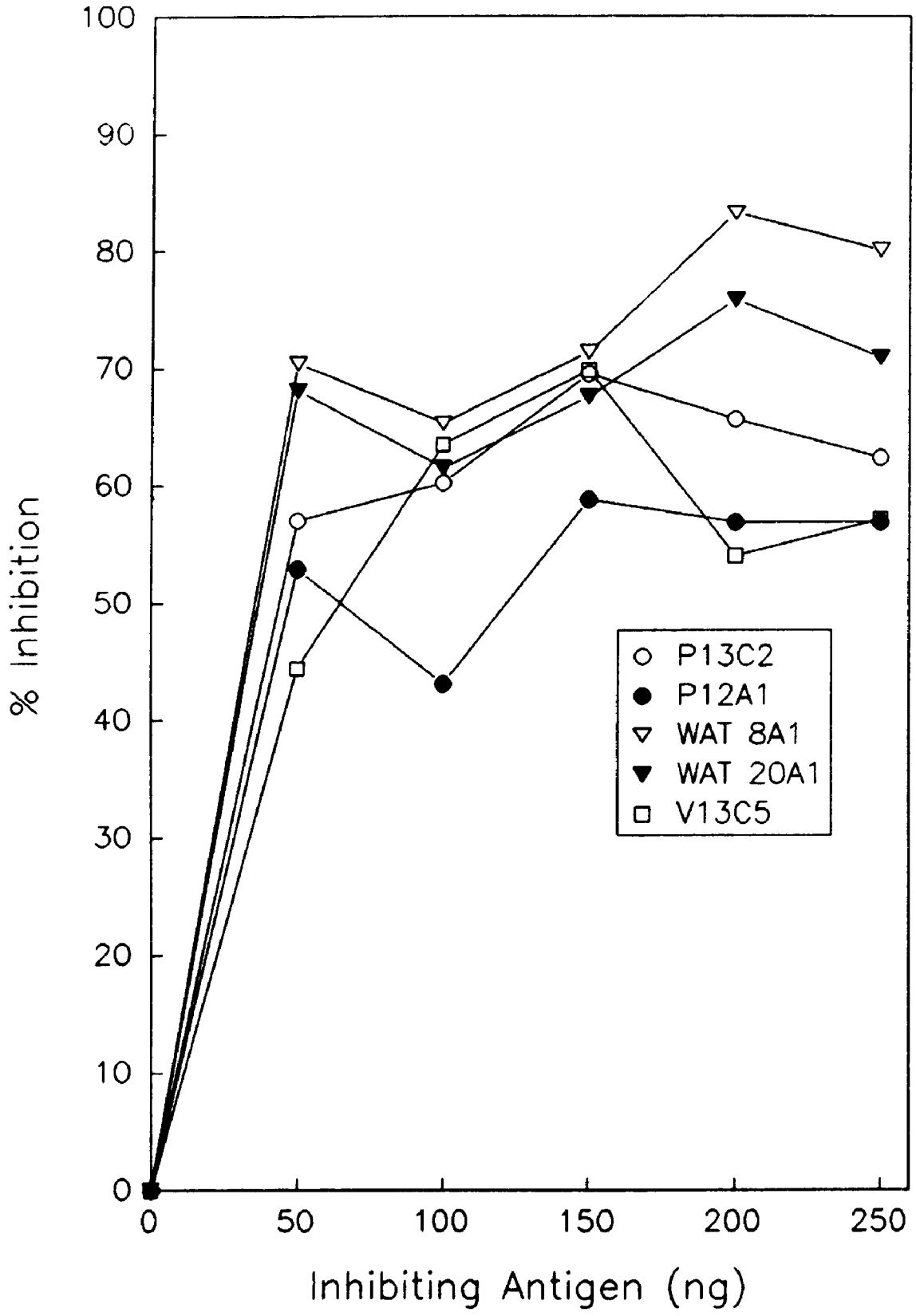
sVSG in an ELISA assay, this does not necessarily suggest that the epitopes to which they bind are exposed on the surface of the native protein because the degree of structural perturbation occurring upon binding of a antigen to a microtitre plate is unknown. Surface exposure or solvent accessibility of antigenic determinants can, however, be determined using an assay based on the ability of antibody to bind to solution phase antigen. If antigen/antibody binding successfully occurs in solution, antibody is prevented from binding to solid phase antigen. Subsequent steps designed to detect the amount of antibody bound to solid phase antigen measure the degree to which inhibition has occurred. By including a range of inhibiting antigen concentrations, one can generate inhibition curves for antibodies which demonstrate the effect.

When this analysis is performed with anti-VSG MAbs, five out of ten tested demonstrated the ability to be inhibited by homologous antigen (Figure 4.15). These include P13C2, P12A1, WAT 8A1, WAT 20A1 and V13C5. Other MAbs included in the assay, not exhibiting inhibition were: P14A1, P14B1, P13A2, P12B1 and P12C1. Profiles for the latter group are not displayed in Figure 4.15 because they demonstrated large enhancing effects (ie. negative inhibition) making their results difficult to convert into percentages and to graph. It was important to conduct this experiment because considering the form of antigen (fragmented) used to immunize mice from which many of the MAbs were derived, solvent accessibility could not be assumed for antigenic sites under study. This information is useful for making conclusions regarding the positions of epitopes on the VSG

Figure 4.15: Inhibition curves generated from solution phase antibody inhibition assays using homologous VSG. % inhibition vs the amount of inhibiting antigen in nanograms (ng) is graphically displayed for 5 anti-VSG MAbs. 5 other MAbs, included in the assay but did not demonstrate inhibition were: P14A1, P14B1, P13C2, P12B1, and P12B1. Assays were performed using WaTat 1.12 sVSG as described in section 4.2.14. % inhibition values were calculated from the formula:

$$\frac{O.D._x}{O.D._0} \times 100 - 100 = \% \text{ inhibition}$$

where $O.D._x$ equals the observed ELISA optical density value for x ng inhibition antigen and $O.D._0$ equals the value for 0 ng inhibiting antigen.


100
90
80
70
60
50
40
30
20
10
0
% Inhibition
0
50
100
150
200
250
Inhibiting Antigen (ng)
P13C2
P12A1
WAT 8A1
WAT 20A1
V13C5

three dimensional structure, as presented in the following section.

4.3.2.8 The Locations of Epitopes on the VSG Three Dimensional Structure

Recently, the 2.9 Angstrom resolution crystal structures of two unrelated VSGs have been solved (Freymann *et al.*, 1990; J. Down, personal communication). The results are no less than astonishing. Although sequence alignment demonstrates that the two VSGs, ILTat 1.24 and MITat 1.2, have identical residues at only 13% of the amino acid positions (Appendix II), their three dimensional structures are almost identical. It should be noted that only the N-terminal domain, which comprises approximately 60% of the entire VSG, is illustrated. The structure consists mainly of two long antiparallel alpha helices surrounded at various points by smaller helix motifs and one area of three short beta sheets near the top of the molecule. There is also one particularly long stretch of random structure which extends from the end of the second long alpha helix, around the core helices and loops across the top of the structure, finally emerging into the beta sheet motif.

Given the remarkable tertiary structure similarity of ILDat 1.24 and MITat 1.2 VSGs, it appears likely that most if not all VSGs, including the WaTat VSGs examined in this study, maintain a highly conserved three dimensional structure. Additional evidence for this idea, in the form of conserved structural motifs, has been reported (Freymann *et al.*, 1990). In order to demonstrate that WaTat VSGs, variant 1.14 in particular, hold no less sequence similarity to, for example, MITat 1.2 than MITat 1.2 holds to ILDat 1.24, another sequence alignment was performed between WaTat 1.14 and MITat 1.2 VSGs (Appendix III). Results show the WaTat

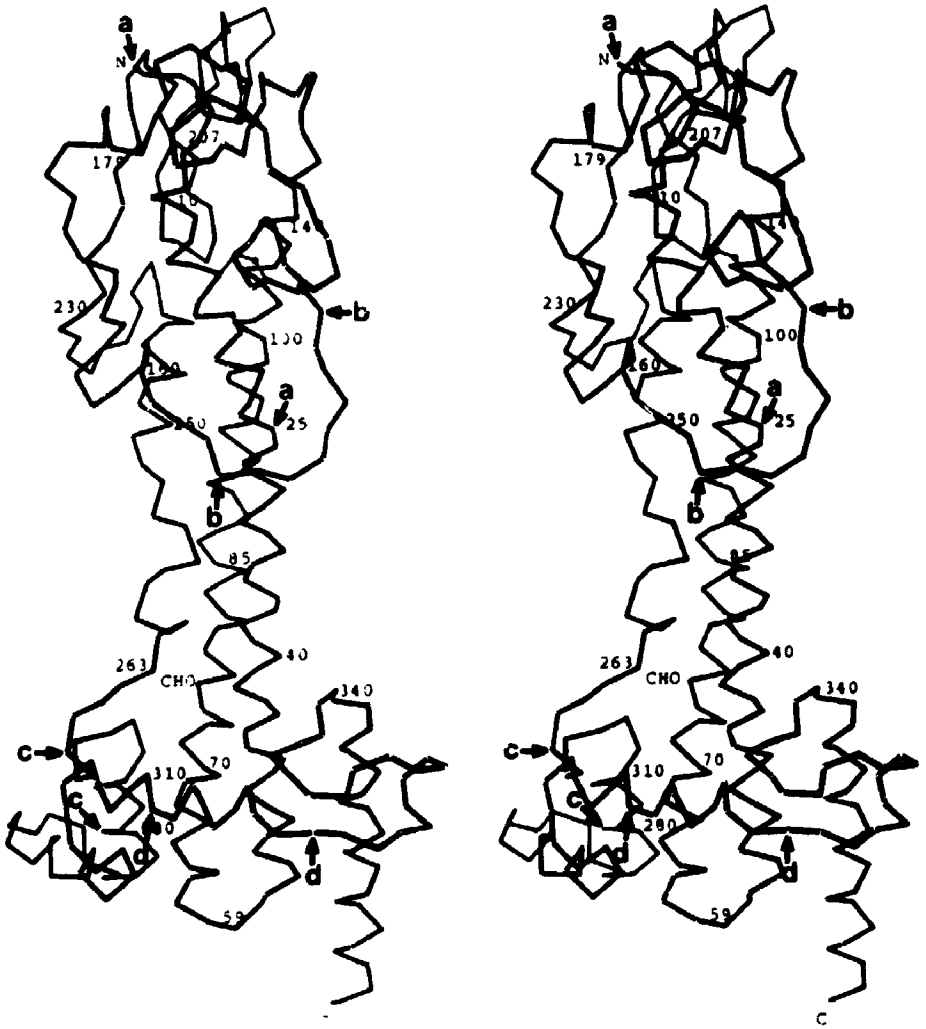
1.14 sequence has even greater similarity to MITat 1.2 than does ILDat 1.24 (21% vs 13% identity). Also and more importantly, the WaTat 1.14 sequence shares essentially all of the structural motifs (highlighted in Appendix III) suggested by Freymann *et al.*, (1990) to be responsible for tertiary conservation in most VSGs. This justifies the initiative of placing the positions of antigenic sites on the MITat 1.2 structure as if it were the structure of the WaTat 1.14 sVSG, provided one keeps in mind that minor structure dissimilarities may exist.

Results, presented in Figure 4.16, demonstrate that P12A1 binds to the end of a short alpha helical segment residing near the bottom of the N-terminal domain. Being a surface-exposed epitope, hydrophilic residues existing on the outside face of the helix probably interact directly with the antibody paratope. This hypothesis could easily be tested with site directed mutagenesis of the hydrophilic residues in the antigenic determinant.

V13C5 (and likely V13B1) appears to bind to a random structure segment also near the bottom of the N-terminal domain. Since this is also a solvent exposed epitope, the antibody presumably interacts with residues having their side chains oriented towards the external environment. However, the possibility of interacting with one or more backbone atoms, in addition to side chains, cannnot be discounted.

P13A2 also binds to a random structure segment that loops around the core alpha helices. This MAb does not recognize its epitope in solution, suggesting contact residues are inaccessible when the VSG is in its "native" conformation.

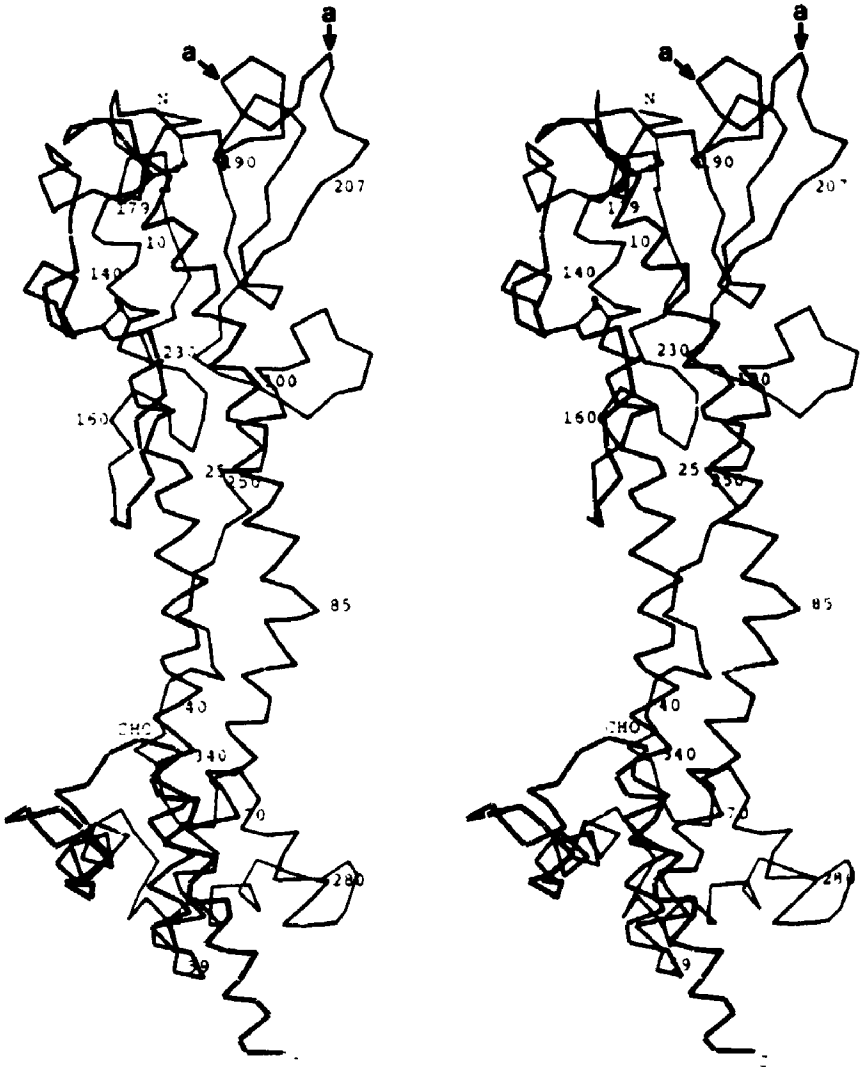
Figure 4.16: Positions of antigenic determinants on the VSG three dimensional structure. The limits of WaTat VSG epitopes modelled on the N-terminal domain crystal structure of MITat 1.2 VSG (modified from Freymann *et al.*, 1990) are indicated by arrows and letters according to the following key: a, P14A1/P14B1; b, P13A2; c, V13B1/V13C5 and d, P12A1. N, mature N-terminus. C, amino acid 361. Justification for using the MITat 1.2 structure as a model is given in section 4.3.2.8.


a
N
179
207
230
100
a
25
b
85
263
CHO
40
140
c
310
70
d
59
C

P14A1 and P14B1 recognize an epitope in the N-terminal 25 amino acids but the site more likely resides within the first 10 or 15 amino acids, judging from evidence for the N-terminal-most border which exists in the first 4 amino acids. This site consists partially of a random structure segment (residues 1-7 or 8) and the first 2 or 3 turns of the first core alpha helix. Inability of P14A1 and P14B1 to bind to VSG in solution correlates with location of their epitope(s) ie. the site is inaccessible because it is buried in the core of the molecule.

The most interesting of all antigenic positions placed on the VSG structure is the site to which P13C2 binds (Figure 4.17). Since, in the trypanosome coat, the C-terminal domain of the VSG is anchored into the membrane, it is assumed that the N-terminal domain is oriented towards the external environment. Given the tight packing arrangement of VSG molecules in the coat, this suggests that the top portion of the molecule is the only area accessible on the VSG when it is assembled into the surface coat. The fact that none of the MAbs used in this study bind to the surface of living trypanosomes supports this postulate. However, included among the surface non-reactive MAbs is P13C2, yet its epitope clearly lies in a random structure segment on the top face of the molecule. Furthermore, from inhibition assay results, the antigenic site to which P13C2 binds must be accessible on the surface of the molecule. Implications of these observations are considered in the following section.

Figure 4.17: Position of the antigenic determinant recognized by P13C2 on the VSG three dimensional structure. The limits of the antigenic site are indicated by arrows and the letter "a". Illustrated is same structure shown in Figure 4.16 rotated 90° around its vertical axis.


a
a
N
190
207
10
140
100
160
85
40
CHO
340
70
280
a
a
N
190
10
140
230
160
85
40
CHO
340

4.4 Discussion

Results from investigations of relative and definitive locations of antigenic determinants on VSG have been presented in this chapter. One sub-pan specific epitope and five pan specific epitopes have been mapped to regions of 6-45 amino acids on the VSG primary structure. Comparison of isoVAT VSG sequences reveals the presence of multiple substitutions occurring in antigenic regions, some of which affect antigenic specificity. Results were generated using monoclonal antibodies by comparing immunoreactive fragment patterns on western blots and by deletion analysis of rVSG expression products. In addition, solvent accessibility of antigenic sites was assessed using a solution phase binding assay. Results indicate half of the antigenic sites recognized by monoclonal antibodies are not solvent exposed. Positions of most antigenic sites have been determined on the VSG three dimensional structure using the recently solved crystal structure of a heterologous VSG as a model (Freymann *et al.*, 1990).

Amongst a total of six substitutions occurring in five pan specific antigenic determinants, the most radical replacements observed involve proline residues in sites recognized by P14A1 + P14B1 and V13B1 + V13C5. These substitutions occurred at central positions in both determinants. Replacements involving proline residues are considered radical because the unique structure of proline tends to significantly alter conformation by putting a kink in the alpha carbon backbone of the polypeptide. Even if proline or the substituted amino acids are not contact residues, replacements of this nature should, theoretically, interfere with the fit

between the antigenic determinant and the antibody paratope. Under the conditions used to determine specificities (ELISA and western blot), no significant differences in antibody binding were observed for isoVAT VSGs, regardless of the amino acid present in proline substituted positions. However, since dissociation constants were not determined, the effects of these substitutions on antibody affinity remains unknown. Furthermore, the degree of structural perturbation created by these proline substitutions is completely unknown. Forthcoming crystal structures of WaTat VSGs should help to resolve this issue. Other allowable substitutions observed involved Gln to Glu (3 positions in 2 determinants) and Gly to Ser (1 position) replacements. Glycine and serine are both small, non-charged amino acids, therefore this substitution is considered conservative. However, glutamine is a neutral amino acid while glutamic acid is negatively charged at physiological pH. This charge discrepancy apparently does not affect specificity, perhaps suggesting that these residues are not in direct contact with the paratope. Further testing, however, is clearly required to confirm this suggestion.

Only one sub-pan specific antigenic determinant, recognized by P12B1 and P12C1, was localized in this study. The antigenic region was mapped to the last 45 amino acids of the C-terminus. Assuming it is linear, the epitope likely does not encompass the entire 45 amino acid region. Inability to define the site further stemmed from difficulties in creating 3'-5' deletions in pGEMEX.12 and from the non-availability of antigenic sites closer to the C-terminus (ie. for use in a differential screen). Nevertheless, since P12B1 and P12C1 do not recognize

variants 1.13 and 1.14, some suggestions regarding critical substitutions can be made. In the C-terminal 45 amino acids substitutions are much more limited in WaTat 1.13 compared to WaTat 1.14. Nevertheless, these replacements are capable of disrupting binding specificity, therefore, they are non-allowable substitutions. At the only positions where both WaTat 1.13 and WaTat 1.14 differ from WaTat 1.1 and WaTat 1.12, Thr to Lys/Ser, Gly to Asp and Lys to Glu/Thr substitutions are observed. Each of these replacements represent significant charge differentials, therefore they are considered radical. Although further experimentation is clearly required, these observations serve as a basis upon which this site can be examined in greater detail.

As a result of this study, potentially immunodominant antigenic regions of the VSG have been disclosed. This is suggested from the observation that independently isolated MAbs were found to recognize the same site in four instances. They include two sites at the extreme termini (P14A1/P14B1 and P12B1/P12C1) and two centrally located determinants (V13B1/V13C5 and WAT 8A1/WAT 20A1). The significance of these observations however is not clear, since immunogenicity is not an intrinsic property of the antigen, therefore, it may be of little relevance to discussion of VSG structure.

The modern molecular biologists arguably faces one problem above all others; he or she can now derive protein sequence information from cloned cDNAs or genes isolated with relative ease but is often left in a position of absolute ignorance regarding the higher ordered structural characteristics of the protein

under study. This predicament provided protein chemists and computer programmers with the incentive to develop methods of predicting secondary and more recently, to some extent, tertiary structure features from primary sequence information. Although a number of computer programs are now available for making these predictions, they are clearly still in the infancy stage of development as evidenced by the relatively low correlations found between the results they generate compared to experimental evidence collected on test proteins (Schulz, 1988). The prediction profile generated by the GCG program (Devereux, 1984), correlates moderately well with the positions of antigenic regions demarcated in this study. This provides some measure of evaluation for computer programs designed to predict antigenic regions and further indicates the necessity for using some caution when making conclusions based on antigenic sites predicted from at least one computer program (Devereux, 1984).

Results of inhibition assays suggest that some MAbs bind to solvent accessible antigenic determinants while others recognize buried or inaccessible sites when the VSG is in solution. Since many of these MAbs were prepared against VSG fragments, recognition of buried sites is not surprising. However, all VSG determinants can be recognized by their respective MAbs when examined using a solid phase ELISA assay. Therefore, some determinants are not accessible on VSG when in solution yet become accessible when the antigen is bound to a microtitre plate. Clearly, significant structural alterations to the VSG result from interaction with the wells of a microtitre plate. Although others have also made this

observation (Green *et al.*, 1982) it serves to further illustrate the importance of conducting solution phase assays when any conclusions regarding epitope positions are to be made.

None of the MAbs used in this study bind to the surface of living trypanosomes (chapter 3), hence they recognize cryptic epitopes. Cryptic antigenic determinants have been assumed to be inaccessible on the trypanosome surface because of obscuration by closely adjacent VSG molecules (likely in dimeric form) in the coat (Miller *et al.*, 1984a; Savage *et al.*, 1984; Miller *et al.*, 1984b; Masterson *et al.*, 1988). A corollary assumption has been that only the top portion of the VSG is exposed to the external environment. Results of the present study disclose epitope positions on the VSG three dimensional structure and therefore, for the first time, provide experimental evidence to support these assumptions. It was found that five antigenic sites are either buried in the core of the molecule (P14A1/P14B1, P13A2 and P12B1/P12C1) or exist at lateral positions on the VSG structure (V13B1/V13C5 and P12A1)(see also Figure 14.16). These sites are accessible on purified VSG yet cryptic on VSG in the coat, suggesting that association of VSG molecules in the coat is sufficiently close to preclude accessibility of these sites to antibody. However, one site (P13C2) appears to be positioned directly at the top of the molecule. Since this site was shown to be solvent accessible on purified sVSG (section 4.3.2.7), it does not appear to be buried or otherwise obscured on solution phase VSG. This raises a perplexing question: given the position and solvent accessibility of the P13C2 determinant,

why is it not exposed on the surface of living trypanosome? Since the P13C2 determinant is accessible on solution phase VSG but not on *in situ* VSG, clearly structural differences must exist between isolated VSG and VSG associated at the trypanosome surface. Furthermore, since the presently available crystal structure is also based on isolated VSG, how much does it differ from the natural structure of the molecule in the coat? Does this suggest some caution should be used when interpreting the forthcoming computer models of VSG associated in the coat? On the other hand, isolated VSG may reassume a more natural form upon crystallization because it is closely associated in both crystals and coat. Nevertheless, since we cannot presently examine the structure of the VSG *in situ*, these queries may deserve consideration.

In this study, two novel methods of examining relative distribution (fragment westerns) and definitive locations (deletion analysis) of antigenic sites have been presented. Furthermore, results generated from epitope mapping by deletion analysis serve to illustrate the high resolution achievable using this strategy. This method has demonstrated the ability to resolve antigenic sites to as few as 6 amino acids, which is as good or, in most cases, better than any other technique available.

In conclusion, results presented in this chapter advance our current knowledge regarding the dependency of antigenic specificity on primary structure of an antigen and the molecular arrangement of VSG in the trypanosome surface coat. As described in the next chapter, extension of these studies will undoubtedly

further add to our understanding of antibody/antigen interactions and the molecular basis of trypanosome survival in its mammalian host.

Chapter 5 - Summary and Future Prospects

The survival of any organism is dependant on its ability to adapt to changes in environment. For infectious organisms, environment almost exclusively means the belligerent confines of an immunocompetent host. Pathogens often depend on mechanisms such as antigenic variation to evade the effects of host immunity. However, in essentially all cases, the extent of antigenic variation is constrained by the necessity to maintain functionality of antigens. That is, most antigens play an essential role in basic survival and propagation of the pathogen. Therefore, polymorphism can only be expressed until it interferes with the function of the antigen. Although antigenic variation may be limited by these means for most infectious organisms, it still poses a serious threat to the potential effectiveness of vaccines being currently developed. A better understanding is required, at the molecular level, of how antigenic polymorphism succeeds in obstructing immune recognition. Knowledge in this regard may enable us to devise ways of counteracting antigenic diversity as an immunoevasive strategy. VSG may be of use in a model system suitable for examining the effects of amino acid sequence variation on antigen/antibody interactions. This focus remains the primary objective of the present thesis.

Results and conclusions described in this dissertation can be summarized as follows:

1/ Structurally, although differing significantly in M_r, isoVAT VSGs

share considerable homology at the primary sequence level as determined using indirect analytical tests. Presently available amino acid sequences deduced from cDNAs of each isoVAT VSG confirm these findings. These results suggest isoVAT VSGs are closely related but distinguishable at the level of primary structure

2/ isoVAT VSGs share at least one immunogenic surface-exposed antigenic site as determined with polyclonal antibodies. Furthermore, monoclonal antibodies can distinguish between members of the isoVAT, suggesting close antigenic similarity but non-identity. These results are consistent with the structural properties manifested by isoVAT VSGs.

3/ Relative distribution of antigenic sites, determined by fragment westerns, indicate the presence of at least three potentially immunodominant regions of the VSG as evidenced from recognition by more than one MAb.

4/ Amino acid sequences constituting antigenic determinants on isoVAT VSGs differ to varying degrees. Among allowable substitutions, those involving proline are the most noteworthy since they likely result in significant conformational alterations that surprisingly

do not affect specificity. Also worthy of description are charge effect substitutions occurring at several positions within antigenic regions. In addition, all three non-allowable substitutions represented alteration of immunochemical specificity.

5/ The recently solved crystal structure of MITat 1.1 VSG (Freymann *et al.*, 1990), combined with results of definitive localization of antigenic sites on the primary structure of WaTat 1.14 VSG, allowed placement of determinants on the VSG three dimensional structure (Freymann *et al.*, 1990). Since all monoclonal antibodies used in this study do not bind to the surface of living trypanosomes, sites mapped to lateral positions on the VSG tertiary structure are inaccessible presumably due to steric obstruction from neighbouring VSG molecules in the VSG coat. However, one site is located at the top, presumably surface-exposed, portion of the VSG structure. The reason for inaccessibility of this site remains unclear.

In terms of furthering our understanding of the dependency of antigenic specificity on primary sequence, this thesis forms a basis for future detailed investigations. Now that the structure of several antigenic determinants has been precisely determined, comprehensive substitutional analyses can be performed in order to further disclose the nature of allowable and non-allowable substitutions.

Experiments of this kind could involve oligonucleotide cassettes synthesized using multiple nucleotides at each cycle such that, on average, each resulting oligomer contains one substitution. Immunochemical screening of clones expressing the substituted antigenic determinant could select at least allowable substitutions. Alternatively, allowable substitutions could be disclosed using the recently developed epitope library approach (Scott and Smith, 1990). This method involves the immunochemical screening (ie. with MAbs) of a filamentous phage expressing randomly generated sequences six amino acids in length. However, for both strategies, non-allowable substitutions can only be inferred from the inability of an antibody to select certain sequences and since this is unsatisfactory, other direct methods must be engineered.

With current knowledge regarding the effects of antigenic polymorphism on immune recognition it is presently impossible to design vaccines capable of accounting for the adaptability of infectious organisms to host immunity. Since diseases such as AIDS and malaria continue to threaten human health, vaccine design encompassing measures to counteract antigenic variation is becoming an increasingly important focus. However, considering the present rate of progress, this goal may not be realized in the near future.

In terms of contributing to understanding of VSG structure and, particularly, molecular arrangement in the trypanosome coat, results presented in this thesis have been of considerable value. Disclosed for the first time are positions on the VSG that are inaccessible to antibody when the antigen is assembled into the coat.

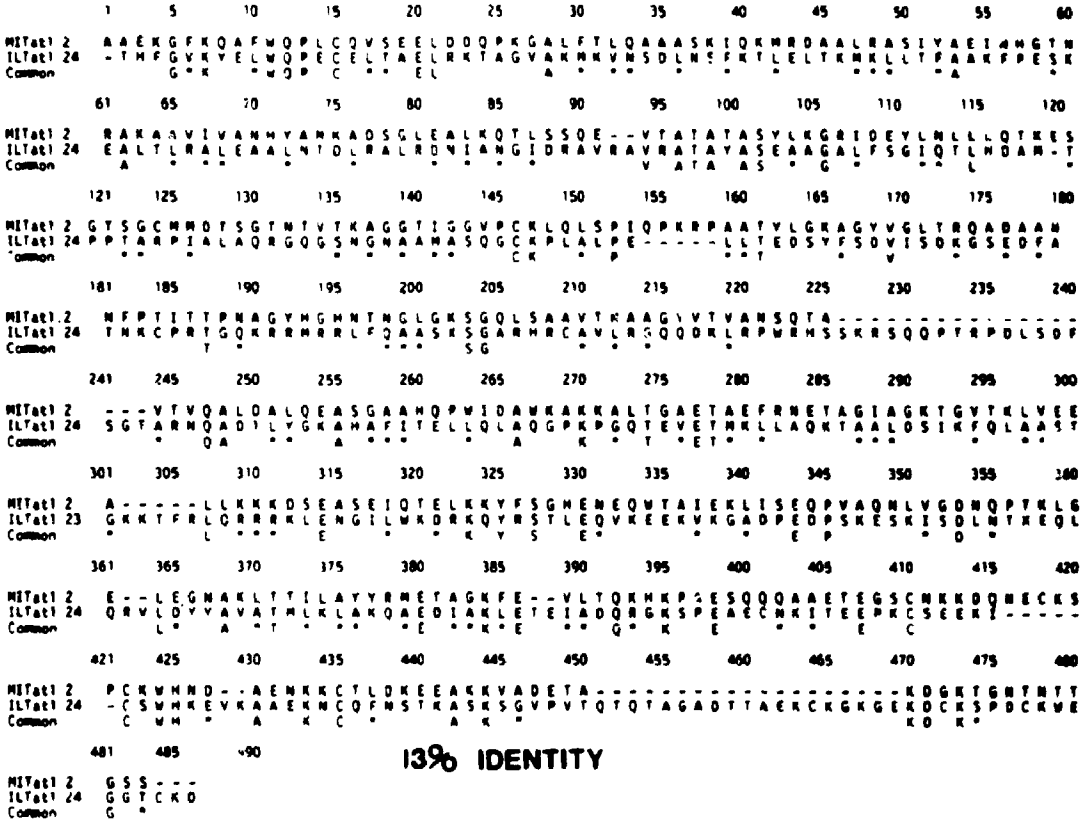
Furthermore, inability of one MAb, P13C2, to bind to a site positioned at the, presumably exposed, top surface of the VSG, suggests the existence of significant structural differences between VSG in isolation and VSG packed in the coat.

Although the present study clarifies our view of the structure of the trypanosome surface coat, further investigations are unquestionably required to advance our understanding in this regard. Accurate measurements of VSG cross-sectional diameter, surface area of the trypanosome and number of VSG molecules/cell are required to determine the average packing density of VSG on the trypanosome. This goal is currently achievable since the first parameter is available and precise measurements of the other two can be made. In addition, computer modelling based on the recently solved VSG structure (Freymann *et al.*, 1990) should reveal the nature of potential interactions between VSG molecules in monolayer. Furthermore, reconstitution experiments using mfVSG in an artificial lipid bilayer may also be of value for examining VSG/VSG interactions. Most insightful, however, will be the results from experiments examining the effects of mutagenesis of surface-exposed amino acid residues on antigenic specificity of determinants accessible on the trypanosome surface. Our laboratory is presently in an optimal position to achieve this goal having developed an easily manipulated system for foreign expression of VSG in its native conformation (Moore and Clarke, unpublished results). This should rapidly allow elucidation of the precise structures recognized by the host immune system and offers hope towards understanding how the trypanosome is so remarkably successful as a parasite.

Appendix I: Multiple sequence alignment of WaTat isoVAT VSGs. The entire coding sequence is displayed for each of the WaTat isoVAT VSGs. Sequences were translated from the sequences of cloned cDNAs and were kindly provided by Dr A.F. Barbet (U. of Florida). The position of the mature N-terminus is indicated by a * and the mature C-terminus by a ^. The entire sequence of WaTat 1.1 is given using the standard single letter codes for amino acids and positions at which the other variants do not differ from the variant 1.1 sequence, are indicated by a -. At positions where the other variants differ from WaTat 1.1, the substitution is indicated by the single letter code of the substituted amino acid. Gaps introduced in the sequences to allow optimal alignment are indicated as a period (.).

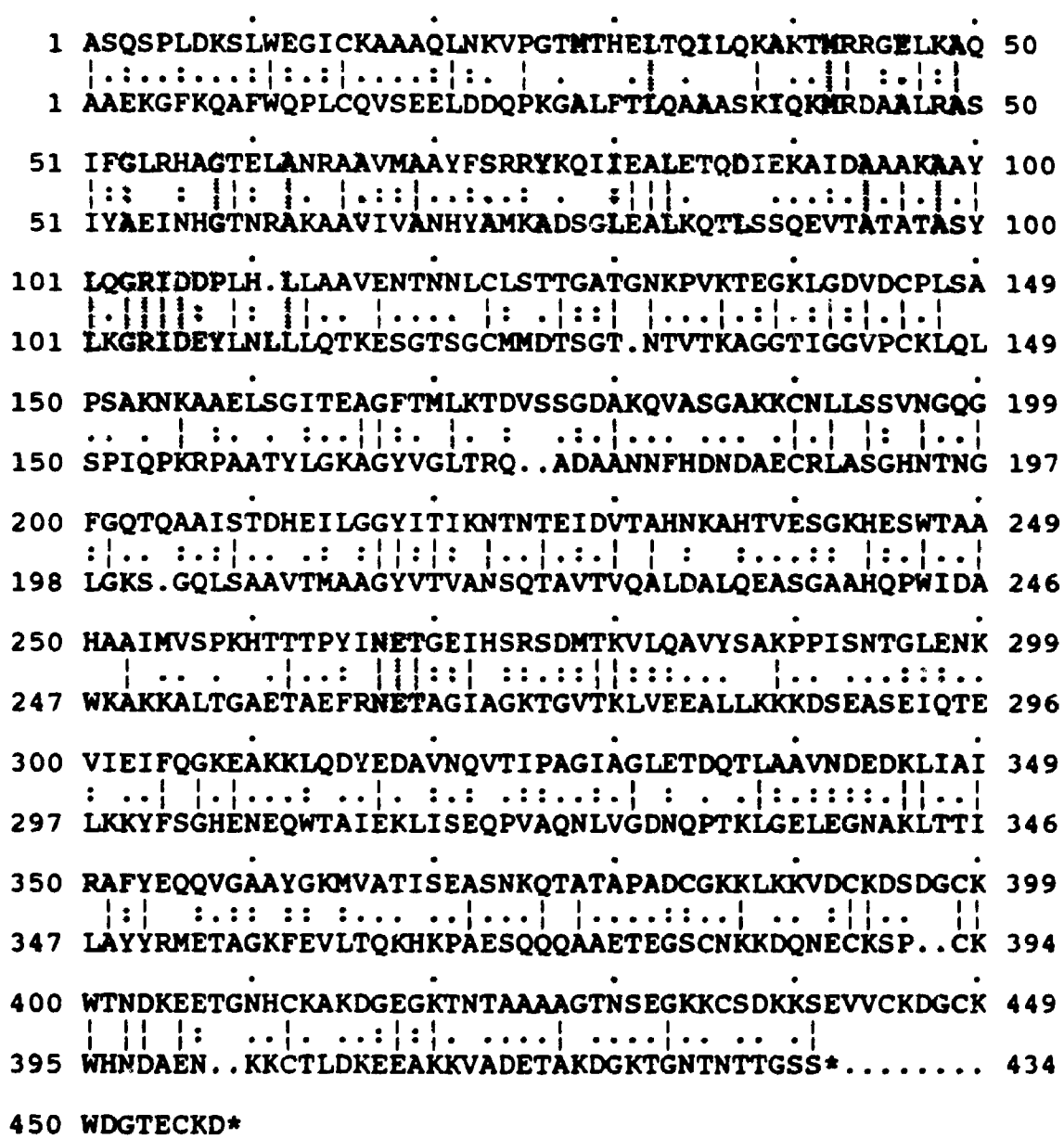
```
1.1                                  MTKSPTKL SQVALSYLLL IAAAPSHVQP
1.12                                 -------- ---------- ----------
1.13                                 ---R--N- NGAM-CS--- L-VS-P----
1.14                                 ---R---- ---------- --VS-P----

          *
1.1       ASQSALDKGL WQGICKATAE LNKVPGTMTH ELTQILQKAK TMRRGELKAQ
1.12      ---------- -E-------- --A------- ---------- ----------
1.13      ----P---S- ---------- ------A--N ------HR-- ----------
1.14      ----P---S- -E-----A-Q ---------- ---------- ----------

1.1       IFGLRHAGTP SANKAAVMAA YFSRRYKQII EALETQDIEK AIDAAAKAAY
1.12      ---------E L-KR------ --G--HN-ML AT---K---- ----------
1.13      ---------- ---------- ---------- ------G--- ----------
1.14      ---------E L--R------ -----T---- ---------- ----------

1.1       LQGRIDDPLH LLAAVDNTNN LCLSTTGATG NKPAKTPGKL GDVDCPLSAP
1.12      ---------- ---------- ---------- ---------- ----------
1.13      ---------- -----E---- ----M---E- ---V--E--- ----------
1.14      ---------- -----E---- ---------- ---V--E--- ----------

1.1       SAKNKAAELS GITQAGFTML KKDVSSGDAK QVASGTKKCN LLSSVNGQGF
1.12      ---------- ---E------ -T--T--N-- -----A---- ------T---
1.13      ---------- ---E------ -T--T--N-- -----A---- ------T---
1.14      ---------- ---E------ -T-------- -----A---- ----------

1.1       GQTEAAISTH HEILGGYITI KDTNTEIDVT AHNKAHTVFS GKHESWTAAH
1.12      -E-------- ---------- -N-------- ---------- ----------
1.13      -E-Q-----D ---------- -N-------- ---------- -------V--
1.14      ---Q-----D ---------- -N-------- ---------- ----------

1.1       AAIHVSPKHT TTPYINETGE IHSRSDMTKV LQAVYSAKPP ISNTGLENKV
1.12      ---------- ---------- ---------- ---------- -TQ-D-----
1.13      ---------- -S--A----- ----P----- ---------- --G-D-----
1.14      ---------- ---------- ---------- ---------- ----------

1.1       IEIFQGKEAK KLQDYEDAVN QVTIPAGIAG LETDQTLAAV NDEDKLIAIR
1.12      ---------- ---------- ---------- ---------- ----------
1.13      --------E- ---------- --------V- ---------- ----------
1.14      ---------- ---------- ---------- ---------- ----------

1.1       AFYEQQVGAA YGKMVATISE ASNKQTATAP ADCGKKLKKV DCKESDGCKW
1.12      -------S-- ---------- ---------- ---------- ----------
1.13      -------S-- --------A- ---------- ---------- ----------
1.14      ---------- ---------- ---------- ---------- ---D------

1.1       NSTDKSEGEF CKPKGEDEQK AQGAGTGDGA AGEQKKEDKC TGKKKDD.CK
1.12      ---------- ---------- ---------- ---------- -------.--
1.13      --------D- --A------- ---------- ---------- KD-----.--
1.14      TN-KEET-NH --A-DGEGKT NTA-.....- --TNSEGK-- SD--SEVV--

1.1       D.GCKWEGKE CKDSSILATK KFALSVVSAA FVALLF
1.12      -.-------- ---------- ---------- ---F--
1.13      SPD-----ET -----F-LN- ----T----- -A----
1.14      -.----D-T- -------L-- ---R------ L-----
                     ^
```

Appendix II: Protein sequence alignment of ILDat 1.24 and MITat 1.2. Modified from Turner (1988). Sequences are given using the standard single letter code for amino acids starting at the mature N-terminus and continuing through to the end of the hydrophobic tail. Conserved amino acids are also indicated by the single letter code for the conserved amino acid and conservative replacements are indicated by an asterisk (*). Gaps introduced to optimize the alignment are marked by a -.



13% IDENTITY

Appendix III: Protein sequence alignment of WaTat 1.14 and MITat 1.2 sVSGs. Sequences are given in the standard one letter code for amino acids with the WaTat 1.14 sequence displayed above MITat 1.2. Two verticle dashes, two spots, and one spot indicate identity, conservative replacement and weakly conservative replacement between two aligned residues respectively. Gaps introduced in order to optimize the alignment are indicated by periods (.). Shown are the mature polypeptide sequences. Conserved structural motifs described by Freymann *et al.*, (1990) are indicated by shaded areas. For description of these conserved structural motifs see section 1.4.


1 ASQSPLDKSLWEGICKAAAQLNKVPGTNTHELTQILQKAKTMRRGELKAQ 50
1 AAEKGFKQAFWQPLCQVSEELDDQPKGALFTLQAAASKIQKMRDAALRAS 50
51 IFGLRHAGTELANRAAVMAAYFSRRYKQIIEALETQDIEKAIDAAAKAAY 100
51 IYAEINHGTNRAKAAVIVANHYAMKADSGLEALKQTLSSQEVTATATASY 100
101 LQGRIDDPLH.LLAAVENTNNLCLSTTGATGNKPVKTEGKLGDVDCPLSA 149
101 LKGRIDEYLNLLLQTKESGTSGCMMDTSGT.NTVTKAGGTIGGVPCKLQL 149
150 PSAKNKAAELSGITEAGFTMLKTDVSSGDAKQVASGAKKCNLLSSVNGQG 199
150 SPIQPKRPAATYLGKAGYVGLTRQ..ADAANNFHDNDAECRLASGHNTNG 197
200 FGQTQAAISTDHEILGGYITIKNTNTEIDVTAHNKAHTVESGKHESWTAA 249
198 LGKS.GQLSAAVTMAAGYVTVANSQTAVTVQALDALQEASGAAHQPWIDA 246
250 HAAIMVSPKHTTTPYINETGEIHSRSDMTKVLQAVYSAKPPISNTGLENK 299
247 WKAKKALTGAETAEFRNETAGIAGKTGVTKLVEEALLKKKDSEASEIQTE 296
300 VIEIFQGKEAKKLQDYEDAVNQVTIPAGIAGLETDQTLAAVNDEDKLIAI 349
297 LKKYFSGHENEQWTAIEKLISEQPVAQNLVGDNQPTKLGELEGNAKLTTI 346
350 RAFYEQQVGAAYGKMVATISEASNKQTATAPADCGKKLKKVDCKDSDGCK 399
347 LAYYRMETAGKFEVLTQKHKPAESQQQAAETEGSCNKKDQNECKSP..CK 394
400 WTNDKEETGNHCKAKDGEGKTNTAAAAGTNSEGKKCSDKKSEVVCKDGCK 449
395 WHNDAEN..KKCTLDKEEAKKVADETAKDGKTGNTNTTGSS*..... 434
450 WDGTECKD*

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